SERUM PERIOSTIN: POPULATION REFERENCE RANGE AND DAILY VARIATION

BY

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A thesis
submitted to the Victoria University of Wellington
in fulfilment of the requirements for the degree of
Masters in Clinical Research

VICTORIA UNIVERSITY OF WELLINGTON

2015

Dr Rachel Caswell-Smith: *Serum Periostin: Population Reference Range and Daily Variation*

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ABSTRACT

Background: Periostin, previously termed Osteoblast-specific factor 2, is an extracellular matrix protein involved in development, stress or injury response, epithelial mesenchymal transition, restructuring of the extracellular matrix and remodelling of tissues. It has been studied across a wide range of tissues and pathologic processes including heart valve and periodontal ligament formation, asthma, myocardial infarction, bone fractures, and atopic dermatitis. It is currently being evaluated as a potential biomarker for directing and monitoring asthma treatment including monoclonal antibody treatment against IL13 and IgE. The clinical utility of periostin as a biomarker is severely limited by the lack of understanding of its serum levels and the factors affecting these levels. The absence of a normal reference range and daily variation may affect its clinical applicability as a TH2 biomarker.

Objectives: To derive age- and sex-related reference intervals from an adult population sample without asthma or COPD and to determine the effect of sampling time on serum periostin levels in adult participants with and without asthma.

Methods:

Reference Range

Serum periostin levels were measured in 480 individuals, comprising 60 female and 60 male adults in each of the 18-30, 31-45, 46-60 and 61-75 year age groups. Key exclusion criteria included a doctor's diagnosis of asthma, chronic bronchitis or COPD, and a history of wheezing or use of respiratory inhalers in the last 12 months. The distribution of periostin and logarithm-transformed periostin levels were derived, and 90% confidence intervals for an individual prediction calculated.

Daily Variation

Serum periostin was measured at 2 hourly intervals from 0800 to 1800 hours in 16 participants with stable asthma prescribed inhaled corticosteroid and long-acting beta-agonist therapy, and in 16 otherwise healthy participants without asthma. Mixed linear models were used to compare time zero (0800) with subsequent measurement time for serum periostin for both asthma and non-asthma groups.

Results:

The distribution of serum periostin was right skewed with a mean (SD) periostin of 51.2 (11.9) ng/ml, median (IQR) 50.1 (43.1 to 56 .9) ng/ml and range 28.1 to 136.4 ng/ml. There was no association between logarithm periostin and age or sex. The 90% confidence limits for periostin were 35.0 and 71.1 ng/ml. There were weak positive associations between logarithm periostin and blood eosinophil count and log IgE, but no association with log FeNO, and weak negative associations with FEV_1, BMI and creatinine clearance.

In both asthma and non-asthma, the mean (SD) serum periostin continuously reduced during the day from 53.5 (13.6) ng/ml at 0800 hours to 50.9 (13.4) ng/ml at 1800 hours (difference log periostin -0.05, P=<0.001) and 50.5 (13.0) ng/ml at 0800 hours to 46.2 (11.5) ng/ml at 1800 hours (difference log periostin -0.08, P=<0.001) respectively. In a post hoc analysis, 1/16 asthma participants changed classification from 'high' (≥50ng/ml) to 'low' (<50ng/ml) periostin, based on 0800 and 1800 hour measurements.

Conclusions: These findings provide reference values for serum periostin levels in adults without asthma or COPD. The weak and inconsistent association between periostin levels and other Th2 biomarkers suggests that they may measure related but different components of the Th2 inflammatory processes. A daily variation in periostin was also established with higher periostin values in the morning compared with the afternoon in both asthmatic and non-asthmatic adults. The small magnitude of the variation in serum periostin levels suggests that the time of day in which the serum periostin measurements are made is unlikely to influence treatment decisions if a specific serum periostin level is used to predict treatment responsiveness.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank to my supervisor Professor Richard Beasley for the valuable insight, enthusiasm, experience and support he has given me throughout this research. I have a tremendous amount of respect for his knowledge and work ethic and am grateful for the opportunity to work with him.

Secondly I would like to thank the Periostin Study team of which there are numerous members. I am extremely grateful for the contributions of this team to this research. Particular thanks goes to Terrianne Cripps and Alexander Hosking for their dedication to the smooth running of the recruitment and visits for the studies, particularly for the work outside my scheduled working hours, and to Thom Charles and Mathew Williams for their patience with me as I learnt spirometry.

My research owes a huge debt to all those mentioned here – Thank you.

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ACRONYMS

ACQ5	Asthma Control Questionnaire (5 question)
Akt/PKB	Protein Kinase B
AQLQ	Asthma Quality of Life Questionnaire
BMI	Body Mass Index
BMP	Bone morphogenetic Protein
BPH	Benign Prostatic Hypertrophy
CD4	T cell expressing Cluster of Differentiation 4 protein
CD8	Cytotoxic T cell expressing Cluster of Differentiation 8 protein
cDNA	Complementary Deoxyribonucleic Acid
CI	Confidence Interval
COPD	Chronic Obstructive Pulmonary Disease
CTU	Clinical Trials Unit
CCDHB	Capital and Coast District Health Board
ECM	Extracellular matrix
eGFR	Estimated Glomerular Filtration Rate
FAS1	Fasicilin 1 Domain
FeNO	Fractional exhaled Nitric Oxide
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
GLI	Global Lung Initiative
GORD	Gastro-oesophageal Reflux Disease
HAQ	Health Assessment Questionnaire
ICS	Inhaled Corticosteroid

IgE	Immunoglobulin E
IL	Interleukin
IL-13	Interleukin 13
IL-4	Interleukin 4
JNK	C-Jun N-terminal Kinases
LABA	Long Acting Beta Agonist
LNCap	Prostate Cancer Cell Line
MI	Myocardial Infarction
MMP	Matrix Metalloproteinases
MRINZ	Medical Research Institute of New Zealand
mRNA	Messenger Ribonucleic Acid
OSF-2	Osteoblast Specific Factor-2
POSTN -/-	Periostin Null Mice
POSTN +/-	Periostin Heterozygous Mice
POSTN	Gene encoding for periostin
PPARα	Peroxisome Proliferation-Activated Receptor α
SAS	Statistical Analysis System
SD	Standard Deviation
TGF β	Transforming Growth Factor
TGF β1	Transforming Growth Factor 1
TGF β2	Transforming Growth Factor 2
TH2	T-helper 2
TSLP	Thymic Stromal Lymphopoietin

Part I

INTRODUCTION AND RESEARCH OBJECTIVES

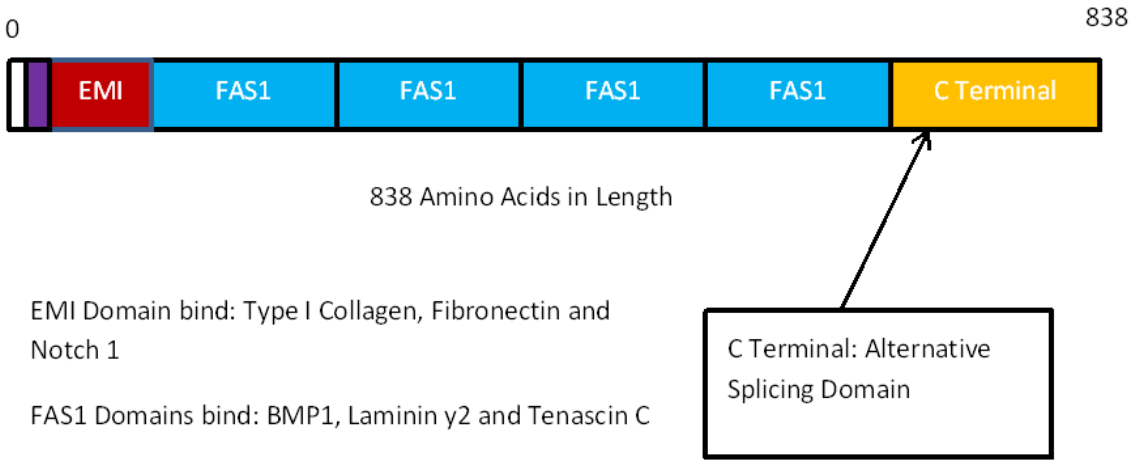
1

INTRODUCTION

Periostin is an extracellular matrix (ECM) protein first described in 1993 after it was isolated from an osteoblast cell line (Takeshita et al. 1993). It was identified using subtraction hybridization and named Osteoblast Specific Factor 2 (OSF-2) after the osteoblast cell line MC3T3-E1 it was isolated from (Romanos et al. 2014; Takeshita et al. 1993). OSF-2 was subsequently renamed periostin in 1999 following the identification of high levels of periostin in developing periosteum, the dense layer of connective tissue surrounding bone (Horiuchi et al. 1999). Periostin is encoded for by the POSTN gene in humans and is found on chromosome 13 (13 q 13.3) (Romanos et al. 2014). It is a 93.3Kd protein and has a number of isoforms produced by alternative splicing in the C terminal domain. Three isoforms were isolated on western blot analysis in 2006; they were 84, 80 and 78Kd in size and although it is postulated that these isomers may have some variance in function this has not been established definitively (Conway et al. 2013; Takayama et al. 2006).

Periostin is approximately 838 amino acids in length and composed of three regions. An N terminal domain approximately 75 amino acids in length which is a cysteine rich EMI domain, named for its homology to the EMLIN family of proteins, a quadruple tandem repeat of the Fascilin1 (FAS1) domains centrally and an alternative splicing domain at the C terminal. It is able to bind proteins through both the EMI domain and all four of the FAS1 domains inducing the action of downstream proteins and playing an essential role in the ordered organization of the ECM (Conway et al. 2013; Sidhu et al. 2010; Takayama et al. 2006).

Figure 0:1 Structure of Periostin



Periostin not only has a role in the organization of the ECM but is seen in many instances of tissue repair throughout the body. It is induced by a number of proteins including transforming growth factor β (TGFβ) 1,2 and 3, vascular endothelial growth factor, bone morphogenetic proteins (BMP) 2 and 4, connective tissue growth factor 2, Interleukin (IL) 3,4,6,13 and Vitamin K. It in turn can bind with itself and other proteins via the EMI domain (fibronectin, type 1 collagen and notch 1) and via the tandem repeat FAS1 domains (BMP-1, tenascin C and laminin y2) and has a downstream modulating effect on multiple genes including sclerostin, fibronectin, type 1 collagen and TGFβ1 (Conway et al 2013; Romanos et al. 2014; Sidhu et al. 2010; Takayama et al. 2006). It has been identified as having multiple effects including; cell proliferation and migration, modulation of elasticity and other biomechanical properties of the ECM and epithelial to mesenchymal transition as seen in wound healing, fibrosis and metastatic disease. It is present in many developmental processes and is often markedly upregulated in response to stress including; mechanical stress, injury, allergens and disease (Bornstein et al. 2009; Conway et al. 2013; Sidhu et al. 2010).

Bone

Early periostin research identified that periostin is present in osteoblasts and in elevated levels in the periosteum surrounding bone (Horiuchi et al. 1999; Takeshiti et al. 1993). In vitro findings indicate that periostin acts on bone primarily through an increase in osteoblast proliferation, differentiation, adhesion and prolonged survival of these cells during embryonic development and times of stress. Inactivation of the POSTN gene using periostin specific antibodies leads to a reduction in osteoblast specific factors including alkaline phosphatase, osteopontin, type 1 collagen, osteocalcin, and an upregulation of sclerostin resulting in poor bone quality and disorganized collagen matrix. This suggests that under

normal conditions a negative feedback effect of periostin on sclerostin occurs in order to preserve bone mass (Bonnet et al. 2009).

It has been demonstrated that after the initial postnatal period where there is a downregulation in periostin levels in the periosteum, there is a re-expression of periostin following bony injury; using cDNA microarray analysis, the POSTN gene was identified in early stages of fracture healing with corresponding high levels of periostin protein found at fracture healing sites, specifically in preosteoblastic periosteal cells and undifferentiated mesenchymal cells. Peak levels of periostin are seen at day seven post fracture and are significantly decreased by day fourteen coinciding with fracture stabilization and periosteal callus formation Periostin has also been demonstrated to regulate collagen cross-linking and fibrillogenesis through the binding of BMP1 via the EMI domain at the N terminal. Under mechanical stress periostin binds to notch 1 prolonging osteoblast survival and affecting osteoblast proliferation (Bonnet et al. 2009; Nakazawa et al. 2004; Conway et al. 2013).

Periostin null mice (POSTN -/-) exhibit growth retardation and in some instances dwarfism, shortened long bones and disrupted epiphyseal plate organization. Microscopic examination of long bones reveals disrupted collagen fibrillogenesis and an increase in sclerostin mRNA on mechanical loading with subsequent sclerostin mediated bone loss. Periostin has also been identified in instances of bone fibrosis including benign bone fibrosis and Scleroderma (Bonnet et al. 2009; Conway et al. 2013; Oku et al. 2008).

Recent results of a seven year prospective follow up study of 607 post-menopausal women (OFLEY study) showed that serum periostin levels were independently associated with increased fracture risk. Women in the highest quartile of periostin levels had a relative risk of fracture of 7.1 (95% confidence interval 2.4-21.8). This result may reflect an increase of periostin in the periosteum, with subsequent secretion into serum, in order to maintain the steady state of bone quality. Compensatory increases in periostin and subsequent bone repair and maintenance may not be sufficient to counteract age/medication related bone loss or increased mechanical stress related to muscle atrophy resulting in a fracture (O'Neal et al. 2014; Rousseau et al. 2014).

Dental

Periostin has been demonstrated to be present in high levels in the developing periodontal ligament and in developing teeth at sites of epithelial-mesenchymal interaction. Expression of periostin is down-regulated postnatally with an absence of periostin noted in erupted teeth. In situations of mechanical

stress and occlusal loading periostin is upregulated and expressed by the periodontal ligament fibroblasts, secreted and then distributed throughout the periodontal ligament extracellular space where it regulates collagen fibrillogenesis and the biomechanical properties of connective tissue around the tooth (Conway et al. 2013; Romanos et al. 2014). Bacterial products and inflammatory cytokines associated with infection have been demonstrated to compromise the function of the periodontium through a reduction in periostin levels and disruption of fibrillogenesis involving type 1 collagen, resulting in loosening of the tooth if the infection is not treated (Romanos et al. 2014).

Periostin null mice (POSTN -/-) exhibit widened periodontal ligaments, abnormal remodelling, destruction of alveolar bone, enamel defects following eruption and a periodontitis-like disorder which is characterized by loss of the periodontal ligament mechanical properties with abnormal tooth migration and premature tooth loss (Conway et al. 2014; Rios et el. 2005;Romanos et al. 2013).

Cardiovascular

Identification of periostin in developing heart valves in mice has led to further study to determine its importance. It has become evident that periostin is essential for normal development of heart valves and the fibrous heart skeleton in utero and that it is downregulated in the postnatal period. Periostin is found in proximity to type 1 collagen and discoidin-domain receptor 2 protein, binding type 1 collagen directly promoting fibrillogenesis within the developing heart. It promotes cardiac stem cell differentiation in fibrogenic lines and functions in an inhibitory capacity to reduce non-fibrogenic differentiation in early cardiac development (Conway et al 2013; Guan et al. 2015; Snider et al. 2008). Within the heart periostin is isolated to fibroblasts/myofibroblasts and is never detectable in cardiomyocytes. In hypertrophic post mortem human cardiac tissue periostin has been identified in cardiac fibroblasts but is absent from adjacent cardiomyocytes. Following cardiac injury, induced by TGF β2, periostin is upregulated and secreted leading to increased serum levels, accelerated mobilization and differentiation of bone marrow cells into cardiac fibroblasts. Periostin has been demonstrated to upregulate expression following myocardial infarction (MI), myocardial hypertrophy, valve calcification and arterial injury. It is induced in response to TGF β1 in vascular smooth muscle cells in vitro and up regulated in vivo in response to pathologic stress/distension of rat arterial walls or direct arterial injury again demonstrating its function in tissue repair (Conway et al. 2013; Guan et al. 2015; Snider et al. 2008).

Periostin null mice (POSTN -/-) exhibit multiple cardiovascular abnormalities. Cardiac valve leaflets are truncated and thickened in all periostin null mice although they are the same weight as normal valves

indicating that the organization of the fibroblasts is affected rather than overall cell numbers. The valves also contain ectopic smooth muscle cells and cardiomyocytes leading to potential twitching and dysfunction of valves. The presence of ectopic cells in the abnormal valves confirms the importance of periostin in the embryonic inhibition of non-fibrogenic differentiation of cardiac stem cells. In the ten days following induction of MI in periostin null mice there are significantly higher rates of ventricular rupture than in controls following MI. In those mice that survive this period without ventricular rupture they exhibit less fibrosis at the area of infarction and significantly better ventricular function in the affected ventricle. In mice with a TGF β2 induced overexpression of periostin no ventricular rupture is seen but these mice develop spontaneous hypertrophy with aging. The presence of periostin in hypertrophic human cardiac tissue and the development of hypertrophy following elevated periostin levels have necessitated research in to the role of periostin in human heart failure and its potential prevention (Conway et al. 2013; Guan et al. 2015; Snider et al 2008).

Skin

Other research groups, based in the United States and Canada, have been examining the role of periostin in the development of normal skin and repair following injury/stress. Immunohistochemistry of both human and murine skin identifies that periostin is localized to the nucleus of immature keratinocytes within the epidermis and to fibroblasts within the dermis. Once keratinocytes have matured and lose their nuclei they no longer express periostin. During embryology and skin development, periostin localizes to the dermis, basement membrane and hair follicles where it interacts with other ECM proteins including collagen, fibrin and fibronectin to provide structural support during skin development. It also acts to modulate proliferation and differentiation of fibroblasts and keratinocytes. Following the development of the skin, in the post-natal period, periostin is down-regulated until times of stress and injury. Although it is present in both fibroblasts and keratinocytes in mature skin it must not be required in copious amounts to maintain skin homeostasis (Jackson-Boeters et al 2009; Zhou et al. 2010).

Following injury to skin periostin is upregulated again demonstrating its role in tissue repair. In a full thickness excisional wound model in mice, periostin is present in granulation tissue at day three following wounding. Increased levels within migrating keratinocytes are seen at the edge of the wound. At day seven post-wounding migrating keratinocyte within the wound exhibit significantly elevated levels of periostin. Periostin is also present in the ECM of the granulation tissue and the remodelling dermis. By day 28 the levels of periostin and its distribution within the skin layers have returned to those demonstrated prior to wounding. Interestingly there is not a significant upregulation of periostin in the dermis following an incisional wound despite marked upregulation following an excisional wound.

The absence of significant periostin upregulation may reflect the minor amount of damage inflicted on the dermis and the presence of healing by first intention rather than healing by second intention with granulation tissue as seen following excisional wounding (Jackson-Boeters et al 2009; Zhou et al. 2010).

Periostin null mice (POSTN -/-) develop thinner skin than controls and microscopy identifies a decrease in the thickness of the dermis of the skin as well as reduced collagen cross linking and abnormal collagen fibrillogenesis (Gordon et al. 2011). Periostin is present in elevated levels in many pathologic processes within skin including; nevus formation, hypertrophic and keloid scarring, as well as atopic dermatitis. It is evident in biopsies of nevi where it is present in the epidermis as in normal skin but also predominantly in the ECM of the remodelled dermis and associated with large fibrils surrounding normal cells. Periostin is abundant in fibrotic skin lesions as seen in scleroderma, and in keloid and hypertrophic scarring (Gordon et al. 2011; Jackson-Boeters et al. 2009; Zhou et al. 2010).

Atopic dermatitis (atopic eczema) a pathologic process involving type 2 inflammation has been shown to involve periostin. Atopic dermatitis features include; aberrant differentiation of fibroblasts and keratinocytes, hyperproliferation with inflammation, and, following chronic inflammation, thickening and scaling of skin. Biopsies of acute inflammation in atopic dermatitis show increased levels of periostin compared to normal skin (Masuoka et al. 2012). Periostin has been demonstrated in vivo to accelerate type 2 inflammation following an allergen trigger, by enhancing the production of thymic stromal lymphopoietin (TSLP) a critical mediator of atopic dermatitis secreted from keratinocytes. In atopic dermatitis allergens trigger first inflammatory reponses including lymphocyte activation with the subsequent release of TH2 cytokines including IL4 and IL13. These cytokines induce the production of periostin within the dermis; periostin in turn acts of keratinocytes to induce proliferation and production of TSLP via $\alpha_v$intergrin. Antibody treatment targeting interleukins released during the atopic dermatitis inflammatory process and partial antagonists for periostin have become the focus for ongoing research into the treatment of severe refractory atopic dermatitis (Shiraishi et al. 2012; Masuoka et al. 2012; Zhou et al. 2010).

Cancer

Periostin has been identified to be overexpressed in a number of cancers. These include: oral cancer, thymoma, breast cancer, non-small cell lung cancer, colon cancer, pancreatic cancer and ovarian cancer (often isolated from ascites related to ovarian cancer). Periostin has been shown in vitro in both human and mouse cancer cell lines, and in vivo, to stimulate cancer growth by preventing cell

apoptosis enhancing cell survival through the protein kinase B pathway (Akt/PKB) via $\alpha_v\beta3$ integrin binding and activation. Periostin also promotes cell differentiation and angiogenesis within tumours and plays a significant role in invasion and metastasis of cancers. Invasion of cancer occurs as a result of epithelial-mesenchymal transition (EMT) of which periostin is driver. Periostin induces a series of matrix metalloproteinases (MMPs) including MMP-9, MMP-10 and MMP-13 which degrade the ECM resulting in invasion and potential metastasis. Retrospective analyses of studies into both breast and colon cancer have identified that higher levels of periostin are associated with a trend towards metastasis (Bao et al. 2004; Conway et al. 2013; Sun et al. 2011).

A human colon cancer cell line with known low metastatic potential was induced to over express periostin in vitro. The overexpression of periostin led to prolonged survival of cancer cells, reduced apoptosis and an increase in migration and metastasis (Bao et al. 2004).

More recent research into prostate cancer has shown that periostin is present in both benign prostatic hypertrophy (BPH) and some prostate cancer cell lines including LNCap a metastatic cell line isolated from a supraclavicular prostate cancer metastasis. Similar levels of periostin were present in epithelial cells in BPH and prostate cancer but a 9.1 fold increase in periostin was seen in stromal cells of prostate cancer compared to BPH. An incomplete inhibition (80% reduction) of periostin in the LNCap prostate cancer cell line inhibited both proliferation and migration of cells presenting periostin as a potential target in the treatment in some cancers including prostate cancer (Sun et al. 2011).

Metabolic

An emerging area of research involving periostin is the examination of its role in the development of obesity, hepatosteatosis (fatty liver) and type II diabetes. Although the majority of this research is in early stages it has been established that periostin is present in elevated levels in adipose tissue of humans with obesity and obese mouse strains including *Psammys obesus*. There is an increased deposition of periostin in visceral adipose tissue when compared to subcutaneous adipose tissue in both human and mouse tissue. Periostin is upregulated in liver tissue samples of obese mice and humans with fatty liver disease. POSTN heterozygous mice (POSTN +/-) show a 41% reduction in periostin levels in liver tissue compared to controls. Although these mice achieve similar levels of obesity at maturity compared with controls, they have significantly lower weight livers with reduced triglyceride content. Periostin promotes the deposition of fat within the liver through C-Jun N-terminal Kinase (JNK)-mediated suppression of fatty acid oxidation and downstream regulation of peroximase proliferator-activated receptor α (PPARα). High serum glucose levels have been shown to upregulate periostin expression indicating a relationship between periostin and diabetes which warrants further quantification (Bolton et al. 2009; Lu et al. 2014; Wu et al. 2014).

Renal

Another emerging area of research into periostin is its involvement in renal development and repair following injury. It has been identified that periostin is induced during nephrogensis primarily by BMP4, a protein also associated with cardiac development (Conway et al. 2011; Sorocos et al. 2011). Periostin expression has been observed in mesenchymal tissue surrounding developing kidney and ureters, as well as renal stroma, epithelial cells of developing ureters and within developing nephrons, with a possible role in smooth muscle cell migration and inhibition of ureteral branching (Satirapoj et al. 2012; Sorocos et al. 2011). Following renal development periostin expression is downregulated; it is not observed to be expressed in the renal tubules of adult kidneys but is seen to be present in small amounts in the arteries and arterioles within the kidney (Guerrot et al. 2012). As with many other tissues in the body it is upregulated following insult or injury. Following renal injury immunohistochemistry staining of periostin increases in intensity and distribution over time within renal tubular cell cytoplasm and in renal tubular cells shed into renal tubular lumen (Satirapoj et al. 2012; Sorocos et al. 2011). It has also been identified to be upregulated as much as 18 fold in the media and adventitia of renal blood vessels in response to prolonged hypertension and the development of hypertensive nephropathy (Guerrot et al. 2012). A strong association between the upregulation of periostin in renal blood vessels and creatinine $r=0.68$, $p<0.001$, proteinuria $r=0.71$, $p<0.001$ and renal blood flow $r= -0.64$, $p<0.001$ has been established introducing periostin as a potential marker of hypertensive renal disease (Guerrot et al. 2012).

Periostin has also been identified in cysts which occur in polycystic kidney disease predominantly within the interstitium of the basal surface of the cysts, and may accelerate cyst growth and promote remodelling of the interstitium.

In a normal developed renal tract periostin is not expressed in measurable quantities and is undetectable in the urine. Following renal injury periostin can be measured in urine with excretion increasing over time particularly following chronic insult as seen in polycystic kidney disease. Current research has not definitively determined the mechanisms through which periostin enters the urine, but postulated methods include; secretion of periostin directly into the lumen from activated renal tubular epithelial cells or the degradation of sloughed real tubular epithelial cells while in the lumen, with subsequent release of periostin. Both proposed mechanisms identify urinary periostin as indicative of distal renal tubular cell injury and as such, it is a promising biomarker for this (Satirapoj et al. 2012; Sorocos et al. 2011).

Respiratory Disease

Perhaps the most extensive research into periostin to date has been conducted in the area of respiratory diseases. The primary focus of this research has been the role of periostin in the inflammation and repair of airways following insult and injury, particularly in T-Helper 2 (TH2) or type 2 eosinophilic asthma and the development of subepithelial fibrosis. Periostin null mice (-/-) show grossly normal airways and no significant impairment in lung function indicating that the role of periostin in lung development in minor compared with its role in airway repair. Periostin has been demonstrated to be present in high levels in airway epithelial cells of patients with TH2 mediated asthma but absent in the epithelial cells of non-asthmatic airways indicating that periostin has little to contribute to the homeostasis of airways and a significant role in airway remodelling and repair. The repair mechanisms that periostin establishes following allergen introduction or injury may exist far beyond the initial insult and contribute to the sustained or chronic inflammation in airways, indeed periostin in its role as a matricellular protein establishes a 'vicious cycle of inflammation and remodelling' (Matsumoto et al. 2014; Takayama et al. 2006).

Like the role of periostin in TH2 mediated inflammation in skin as seen in atopic dermatitis (atopic eczema), periostin is secreted in response to TH2 mediated pathways in asthma. A subset of asthmatic patients develop sustained inflammation in their airways as a direct response to TH2 mediated pathways. Allergens in the airways activate and recruit TH2 helper lymphocytes (CD^4 and CD^8 lymphocytes) which once activated produce inflammatory cytokines including interleukins. These interleukins, predominantly IL13 and IL4, drive an inflammatory process which leads to epithelial goblet cell metaplasia with increased mucin production, altered epithelial mesenchymal signalling with subsequent subepithelial fibrosis and hyperplasia of smooth muscle affecting the distensibility of the airways. Periostin is induced by IL-13 released from activated lymphocytes; it is then released from activated airway epithelial cells basolaterally into the underlying ECM. Within this matrix periostin interacts with other matricellular proteins including type I collagen, tenascin C, and fibronectin enhancing fibrosis within the airways. Periostin also assists in eosinophil recruitment and infiltration to sites of TH2 mediated inflammation in the airways and eosinophil-mediated fibrosis through increased eosinophil motility and adhesion at the site of inflammation. Periostin released basolaterally into the ECM can also activate Type I collagen production in airway fibroblasts through TGFβ mediation and increase the elasticity of the ECM altering the biomechanical properties of the airways (Conway et al. 2013; Corren et al. 2011; Sidhu et al. 2010; Woodruff et al. 2007).

Post mortem samples of airway tissue from asthmatics have demonstrated that levels of periostin correspond directly with the thickness of the reticular basement membrane supporting evidence that it is involved in subepithelial fibrosis (Kanemitsu et al. 2014).

Recent studies have established periostin as a promising biomarker in asthma management. Kanemitsu et al. demonstrated that high periostin, particularly over 95ng/ml, is the only factor independently associated with a greater annual decline in lung function (>30ml/year). This study also reported a positive correlation between periostin levels and blood eosinophils (Kanemitsu et al. 2014) Not only does periostin correlate with blood eosinophils it is the single best predictor of airway eosinophil count in patients with refractory asthma despite maximal inhaled corticosteroid (ICS) treatment (>1000 μg/day Fluticasone propionate equivalent). Periostin was found to be superior to fractional exhaled nitric oxide (FeNO), blood eosinophil count and serum immunoglobulin E (IgE) levels at predicting the level of airway eosinophilia seen on bronchoscopy samples (Jia et al. 2012). Periostin may also be able to direct treatment in asthma by indicating which patients will respond best to new therapies emerging on to the market. The EXTRA study, a double blinded randomized control trial of Omalizumab an anti-IgE antibody, involved 850 patients with asthma. Those patients with high serum periostin levels (n=255) showed a 30% decrease in number of severe exacerbations when treated with Omalizumab compared with only a 3% reduction in patients with low serum periostin (n=279) (Hanania et al. 2013). A similar study involving an anti-IL13 antibody Lebrikizumab demonstrated an 8.2% increase in FEV_1 compared to placebo in response to treatment in those with high serum periostin (periostin above the median) with only a 1.6% increase in FEV_1 seen in those with low periostin (below the median) (Corren et al. 2011).

Despite the wide ranging research into periostin at a cellular level, little has been established about the normal serum levels of periostin and the factors affecting these levels. It has been determined that periostin, or pathways involving the induction of periostin, are potential targets for therapies in pathologic processes including; atopic dermatitis, asthma, particularly treatment refractory eosinophilic asthma, and cancer, particularly targeting invasion and metastatic spread. In order to identify those individuals that would benefit most significantly from periostin targeted treatment, a greater understanding of serum periostin and all its modulating factors needs to be gained. If serum periostin is able to be used as a biomarker for directing and monitoring treatment it would provide a much less invasive method than monitoring at a cellular level as seen in bronchoscopy airway sampling to determine eosinophil levels. A single blood test would be a much quicker, cheaper and simpler option than a bronchoscopy procedure as well as being considerably less invasive making it a favourable alternative to both patients and their treating physicians.

The lack of reliable reference values for serum periostin is severely limiting the clinical utility of periostin as a biomarker and the implementation of new antibody based treatments in asthma particularly. This thesis strives to further develop our knowledge of serum periostin by examining it at a population level. The normal reference values for serum periostin in an adult population without respiratory disease are presented along with the daily variation in both those with and without asthma. In addition, this thesis also outlines the relationship between serum periostin and common comorbidities and other markers of TH2 inflammation including blood eosinophil levels, FeNO and IgE levels.

2

RESEARCH OBJECTIVES

In order to determine both the reference range for periostin in a population without Asthma of COPD and the daily variation in periostin, this research has been divided into two related studies which have been reported separately:

Study A: The reference range of periostin in a population without asthma or COPD.

Objectives:

1. To determine age and sex specific reference ranges for periostin in a general adult population without asthma or any other significant respiratory co morbidity
2. To describe the association between serum periostin and specified clinical and pathophysiological markers.

Primary outcome measure:

- Reference range (90% confidence intervals for the general population) for serum periostin

Secondary outcome measures:

Investigation of potential associations between serum periostin and the following:

- Non-asthmatic health conditions (as captured by general health questionnaire e.g. diabetes, hypertension)
- Spirometry (FEV1 and FVC)
- Fractional exhaled Nitric Oxide (FeNO) level
- Full blood count including white cell differential (Eosinophil count)

- Serum creatinine, urea and electrolytes
- Serum IgE
- Ethnicity

Study B: Daily variation in serum periostin in both adults with and without asthma.

Objectives:

1. To determine the effect of sampling time on serum periostin levels in adult participants with and without asthma.

2. To compare the effect of sampling time on serum periostin levels with related TH_2 inflammation measurements of FeNO and peripheral blood eosinophil levels.

Primary outcome measure:

- Effect of sampling time on serum periostin levels.

Secondary outcome measures:

Investigation of potential associations between serum periostin and the following:

- Non-asthmatic health conditions (as captured by general health questionnaire e.g. diabetes, hypertension)
- Respiratory health (as captured by AQLQ and ACQ)
- Spirometry (FEV1 and FVC)
- Fractional exhaled Nitric Oxide (FeNO) level
- Eosinophil levels

Part II

STUDY A

3

METHODS

A cross-sectional study was undertaken in adults aged 18 to 75 years recruited from the Medical Research Institute of New Zealand (MRINZ) volunteer databases and word of mouth. The potential participants had not previously been contacted by MRINZ but had been randomly selected from the electoral roll for a previous study (New Zealand Respiratory Health Survey 2011-12). A one page letter was sent to potential participants inviting them to take part in the study, outlining the study rationale and procedures and providing email, phone and return mail options for accepting or declining the invitation. Once participants had contacted MRINZ accepting the invitation to take part in the study they were contacted by an investigator, phone screened and provided with a patient information sheet. Up to 500 participants were enrolled to ensure that 60 male and 60 female participants were included in each of the pre-specified 18 to 30, 31 to 45, 46 to 60 and 61 to 75 year age groups. Exclusion criteria applied following completion of an investigator-administered questionnaire (appendix 1) included: doctor's diagnosis of asthma; chronic bronchitis or COPD; a history of wheezing or use of respiratory inhalers in the past 12 months; known pregnancy; hospital admission within last 3 months; major surgery requiring general anaesthetic within the last 3 months; dental extractions or root canal procedures in last 3 months; bone fracture within the last 3 months; active (current, or within the 3 weeks prior to the visit) upper or lower respiratory tract infection; significant unstable comorbidities; or any safety concerns at the investigator's discretion. The exclusion criteria were determined based on a literature review of the factors affecting periostin at a cellular level. It has been established that periostin is involved in a number of systems within the body in both a developmental and remodelling capacity. As the effect of stress or injury on serum periostin levels are unknown exclusions were made to remove potential confounders.

Ethical approval was given by the Central Regional Ethics Committee of New Zealand (13/NTB/183/AM01). The trial was prospectively registered with Australian New Zealand Clinical Trials Registry (ACTRN12614000047695) and written informed consent was obtained from all participants prior to testing.

STUDY PROCEDURES

Following telephone or email pre-screening to assess eligibility to participate in the study, participants attended the MRINZ outpatient facility at Wellington Hospital for a single visit. The following procedures were completed: written informed consent, completion of a General Health Questionnaire, measurement of Body Mass Index (BMI), Forced Expiratory Volume in one second (FEV_1) and Forced Vital Capacity (FVC), FeNO, and a blood sample was taken for measurement of full blood count, creatinine, urea and electrolytes, serum IgE, and serum periostin level.

General Health Questionnaire:

The general health questionnaire was used to obtain the current health status of participants, including a history of cancer, cardiovascular disease, gastro-oesophageal reflux (GORD), rhinitis, sinusitis, and eczema (Appendix 1). The medical history was obtained using questions drawn from a series of validated questionnaires including the Health Assessment Questionnaire (HAQ) from Stanford University (Bruce et al. 2003) with co-morbidities recorded using questions from the American Thoracic Society (ATS) Division of Lung Diseases-78 (DLD-78) questionnaire. Following a literature review, questions were formulated to capture information about those body systems where periostin is known to be involved. This information was then used to exclude participants where there may be a confounding illness or injury such as asthma, or bone fracture. Participants with TH2 related pathology other than asthma (atopic dermatitis, eosinophilic oesophagitis and eosinophilic sinusitis) were not excluded from the study but the conditions were well documented during the questionnaire for use in the TH2 related analysis. The questionnaire was administered by doctors and trained investigators only. All investigators were trained by the lead investigator to ensure consistency in the administration of the questionnaire. The justification for the general health questionnaire with references has been provided in Appendix 2.

Lung function and FeNO:

Prior to lung function testing participants avoided eating for one hour, smoking tobacco for two hours, and caffeine ingestion for six hours as recommended in the ATS criteria. (Miller et al. 2005)

Participants were not tested within three weeks of an upper or lower respiratory tract infection as nitric oxide levels have been shown to be elevated during an acute infection but return to baseline within three weeks following the onset of symptoms (Kharitonov et al. 1995; ATS 2005). Expired nitric oxide testing was performed prior to spirometry as spirometric manoeuvers have been shown to significantly reduce FeNO levels initially (1 and 5 minutes post spirometry), returning to baseline values at one hour following spirometry (ATS 2005; Deykin et al. 1998; Silkoff et al. 1999) It is postulated that the drop in FeNO of up to 18% is as a result of bronchospasm induced by repeated spirometric efforts (Silkoff et al.1999).

Expired Nitric Oxide

Expired Nitric Oxide (FeNO) was determined according to ATS guidelines (ATS 2005) with a nitric oxide monitor (NiOX MINO, Aerocrine AB, Solna, Sweden). The testing was performed in a seated position and was explained prior to commencing. Verbal cues and positive encouragement was given throughout the testing. A single test was performed as per manufacturer guidelines and supporting research published following the ATS guidelines in 2005 (Alving et al. 2006; Khalili et al. 2007; Menzies et al. 2007)

Participants exhaled completely and then inhaled ambient air through a nitric oxide scrubber to total lung capacity. They then exhaled against an automatically adjusting resistance to achieve a constant exhalation flow rate of 50 ml/s +/- 10%. Resistance was adjusted automatically so that an upper airway pressure of 10-20 cm H_2O was maintained throughout exhalation, sufficient to close the velum and exclude nasal air. FeNO measurements were taken from a stable plateau in exhaled nitric oxide concentration during an exhalation. Exhalations where flow rate and plateau criteria are not met were deemed not acceptable for measurement. Repeated exhalations were performed a maximum of six times to obtain an acceptable measurement.

Spirometry

Spirometry was performed with measurement of FEV_1 and FVC using a Masterscreen Pneumo (Masterscreen Version 2.0, Carefusion, Leibnizstrasse Hoechberg, Germany) in accordance with American Thoracic Society (ATS) guidelines. All testing was performed in a seating position with nose clips on. Testing was explained to the participant prior to starting and positive encouragement and verbal cues were given throughout the testing.

Acceptability Criteria

Individual tests were acceptable if they were free from:

- Artefacts
- Cough during the first second of exhalation
- Glottis closure that influences the measurement
- Early termination or cut-off
- Effort that is not maximal throughout
- Leak
- Obstructed mouthpiece (e.g. tongue in front of mouthpiece)

They had good starts:

- Extrapolated volume <5% of FVC or 150ml, whichever is greater

They showed satisfactory exhalation:

- Duration of ≥ 6s or a plateau in the volume-time curve or if the subject could not or should not continue to exhale.

Repeatability Criteria

Testing was repeatable if:

- The two largest values of FVC were within 150ml of each other
- The two largest values of FEV1 were within 150ml of each other.

Testing continued until three acceptable manoeuvres had been completed or the subject had performed eight manoeuvres and could not or should not continue with testing. According to ATS guideline participants who were unable to produce reproducible flow volume loops (within 150ml variation in FEV1 and FVC) were not excluded from analysis. In subjects who were not able to produce three acceptable flow volume loops comments regarding technical acceptability of their testing were made. Predicted FEV1 and FVC values were calculated using the GLI criteria which adjust for age, sex, height, weight and ethnicity (Quanjer et al. 2012).

Bloods:

Venous blood samples were drawn with participants in a seated position unless a lying position was requested by the participant. Full blood count and differential (Sysmex platform, Mundelein, USA) and urea and electrolytes (Roche, Cobas 501, NZ) were performed immediately; IgE was performed within 24 hours of blood draw following coagulation and centrifugation (Roche modular, Indianapolis, USA).

Blood samples were coagulated, centrifuged and serum aliquots stored at -80°C, prior to analysis of serum periostin. Serum periostin levels were determined using the clinical trial version of the Elecsys® Periostin immunoassay (Roche Diagnostics, Penzberg, Germany) intended for use on the cobas e 601. The Elecsys® Periostin immunoassay is an automated assay immunoassay is an automated assay based on the sandwich principle and employs two monoclonal antibodies targeted to periostin. The antibodies have previous been described in Jia et al. A sandwich complex is formed between periostin, a biotinylated antibody and a ruthenylated antibody, and is captured on the surface of added streptavidin-coated microparticles. The amount of captured complex and therefore the periostin level in the sample is measured using electrochemiluminescence technology.

Height and Weight:

Height was measured using a wall mounted height rod with shoes removed. Weight was measured on a single Seca electronic scale with outside clothes and shoes removed. The values obtained were then used for calculating both BMI and spirometry predicted values.

STUDY POWER

The sample size was based on the recommendations of the Clinical and Laboratory Standards Institute. The working group recommended that in order to derive a 95% confidence interval 120 reference subjects with a value from each subject are required. (Horowitz et al. 2008).
120 participants (60 female, 60 male) were recruited in each of the four age groups so that if necessary 90% confidence limits could be computed using non-parametric methods (Horowitz et al. 2008; Read et al. 1971).

STATISTICAL METHODS

Simple data summaries are used to describe the participants.

Frequency histograms were derived for periostin and for logarithm (log) periostin. Later in the analysis an outlying value of periostin was identified and frequency histograms were derived with this outlying value removed. The outlying value was removed from the analysis as recommendations of the Clinical and Laboratory Standards Institute state that nonparametrically estimated reference values based on at least 120 participants are not changed at all if the outlying value is removed (Horowitz et al. 2008). Formal tests for normal distribution of periostin, and subsequently the residuals from regression

modelling, were carried out by the four default methods available in the statistical analysis system (SAS) (Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling). The periostin data were skewed even after removal of the outlying value and the log transformation was used.

A simple t-test was used to compare the difference in periostin by sex on the log transformed scale, and regression was used to test for the association with age on the log transformed scale.

Scatter plots were derived with superimposed predicted values together with 90% confidence limits for individual predictions based on an 'intercept only' model and a model with age. The upper limit is that which is predicted to be exceeded by only 5% of the population. Back-transformation of the log predicted values was used to produce the upper confidence limit for a prediction on the scale of measurement. The 95% confidence limits for an individual prediction were also derived. In the event there was no evidence of an association with age or sex and so the upper 90% confidence limit for an individual prediction from an 'intercept only' model was used as the basis for the upper limit of the reference range.

The strength of association between the other continuous variables and periostin was carried out on the log transformed scale. In these models R-square is the amount of variation in log periostin explained by the other variable, which can range from 0 to 100%, and the correlation coefficient is the square root of this, representing the strength of linear association.

The strength of association between the categorical variables and periostin was also carried out on the logarithm transformed scale by t-tests for dichotomous variables and ANOVA for ethnicity.

Statistical Analysis System (SAS) version 9.3 was used.

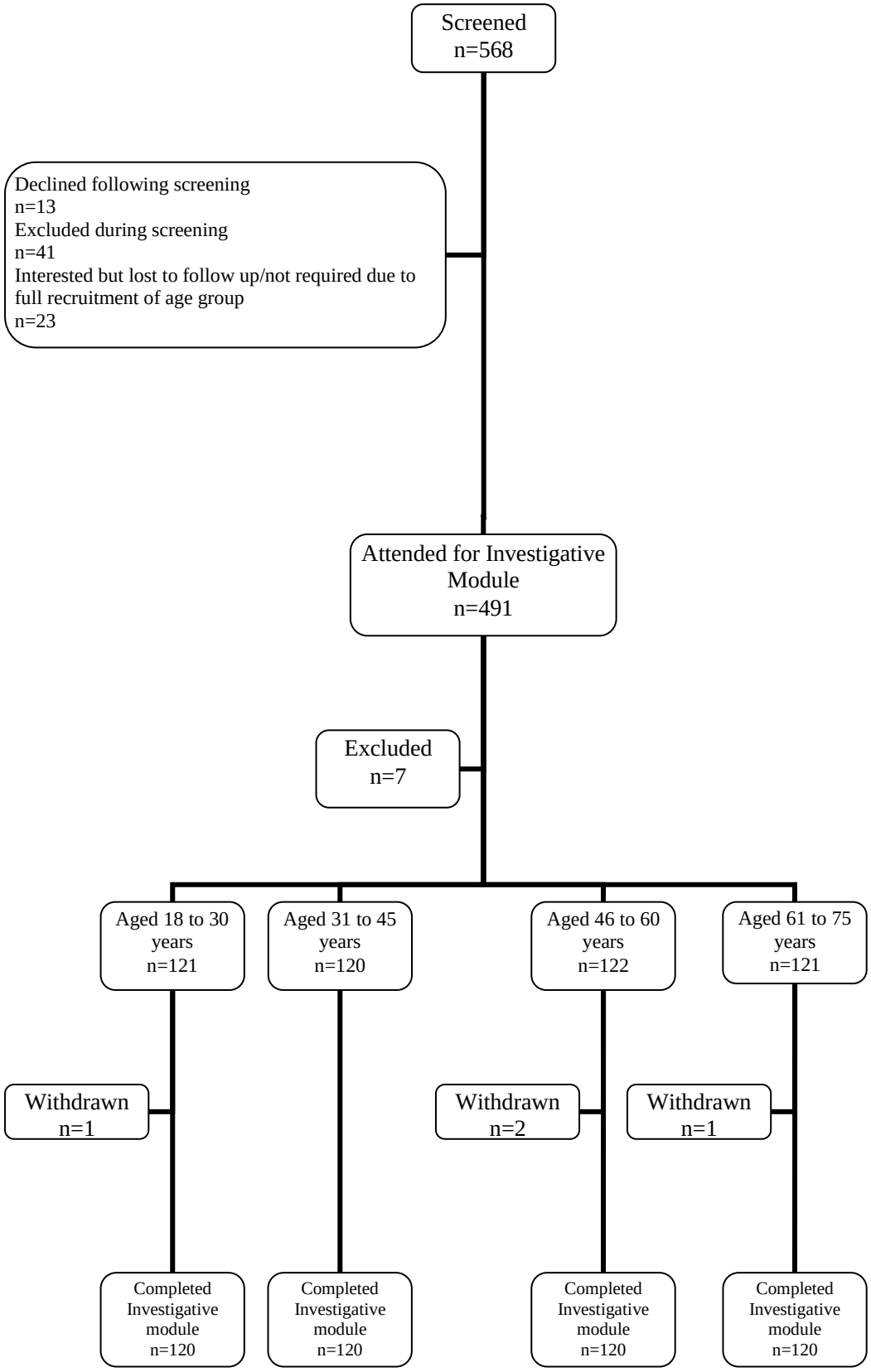
4

RESULTS

Sample description

The flow of participants through the study is shown in Figure 4.1. 2695 potential participants were contacted about this study. There was a 29.3% response rate with 222 choosing to decline to participate. 568 potential participants were screened with 480 completing the study. The characteristics of the participants are shown in Table 4.1. The median FEV_1 was 103.6% predicted, consistent with enrolment of a population sample without respiratory disease. The interquartile range (IQR) for the Th2 biomarkers were within the normal reference ranges for the local laboratory; the highest values for blood eosinophil count, FeNO and serum IgE were 0.8 $x10^9$/L, 300 ppb (the upper limit of the NIOX MINO), and 2608 IU/L respectively. Considering atopy, a doctor diagnosis of eczema was present in 18.1%, with 5% requiring topical eczema treatment (27.6% of those with diagnosis of eczema), hayfever (in the form of allergic rhinitis, eye irritation symptoms or both) in 40.8% with 4.8% requiring antihistamine treatment (11.8% of those reporting symptoms of hayfever) and nasal polyps in 2.7%.

Figure 4.1: Flow of subjects in study



Screening exclusions (41): wheeze in past 12 months (11), dental extraction in last 3 months (6), diagnosis of asthma (4), unwell at visit one – replaced by another participant (4), chronic bronchitis (3), on prednisone (3), fractures in last 3 months (3), surgery in last 3 months (2), hospital admission in last 3 months (2), outside age range (1), post-partum (1), metastatic cancer (1).

Table 4.1: Characteristics of participants (N=480)
a) Continuous variables

Variable	Mean (SD)	Median (IQR)	Min to Max
Clinical:			
Age (years)	45.6 (16.9)	45.5 (30.5 to 60.5)	18.0 to 74.0
Weight (kg)	76.4 (15.4)	74.8 (65.6 to 85.9)	39.7 to 122.9
Height (cm)	170.9 (9.4)	170.4 (164.3 to 177.6)	141 to 198.5
BMI (kg/cm^2)	26.1 (4.7)	25.2 (22.7 to 28.5)	17.1 to 57.5
Creatinine (μmol/L)	78.7 (13.8)	77.5 (68.5 to 87.5)	50.0 to 144.0
Creatinine clearance (ml/min)	105.5 (30.4)	103.5 (82.8 to 121.4)	46.2 to 239
Urea (mmol/L) (N=479)	5.2 (1.3)	5.1 (4.3 to 6.0)	2.3 to 11.7
Haemoglobin (g/L) (N=479)	143.0 (13.9)	142.0 (133.0 to 154.0)	94.0 to 182.0
White cell count ($x10^9$/L) (N=479)	5.9 (1.4)	5.7 (4.9 to 6.7)	2.6 to 12.4
Lymphocyte count ($x10^9$/L) (N=479)	1.88 (0.54)	1.8 (1.5 to 2.2)	0.8 to 4.5
Pack years	2.7 (7.5)	0.0 (0 to 0.65)	0 to 50
Lung function:			
FEV_1 % predicted (N=479)	103.4 (12.8)	103.6 (94.7 to 111.8)	71.3 to 154.3
FVC % predicted (N=479)	108.1 (13.2)	107.1 (99.6 to 116.6)	72.9 to 155.5
FEV_1/FVC % (N=479)	95.5 (7.4)	96.3 (91.1 to 100.5)	60.9 to 115.4
Th2 markers:			
Blood eosinophil ($x10^9$/L) (N=479)	0.16 (0.12)	0.1 (0.1 to 0.2)	0 to 0.8
FeNO (ppb)	24.5 (20.3)	19.0 (14.5 to 27.0)	2.5 to 300
IgE (IU/L) (N=479)	108.7 (257.7)	34.0 (12 to 90)	0.5 to 2608

Laboratory reference ranges: Creatinine (μmol/L) 45-90, Creatinine clearance (ml/min) >89, Urea (mmol/L) 2.4-7.5, Haemoglobin (g/L) 115-155, White cell count ($x10^9$/L) 4.0-11.0, Lymphocyte count ($x10^9$/L) 1.0-4.0, Blood eosinophil ($x10^9$/L) 0.0-0.5, IgE (IU/L) 0-100

Table 4.1: Characteristics of participants (continued)
b) Categorical variables

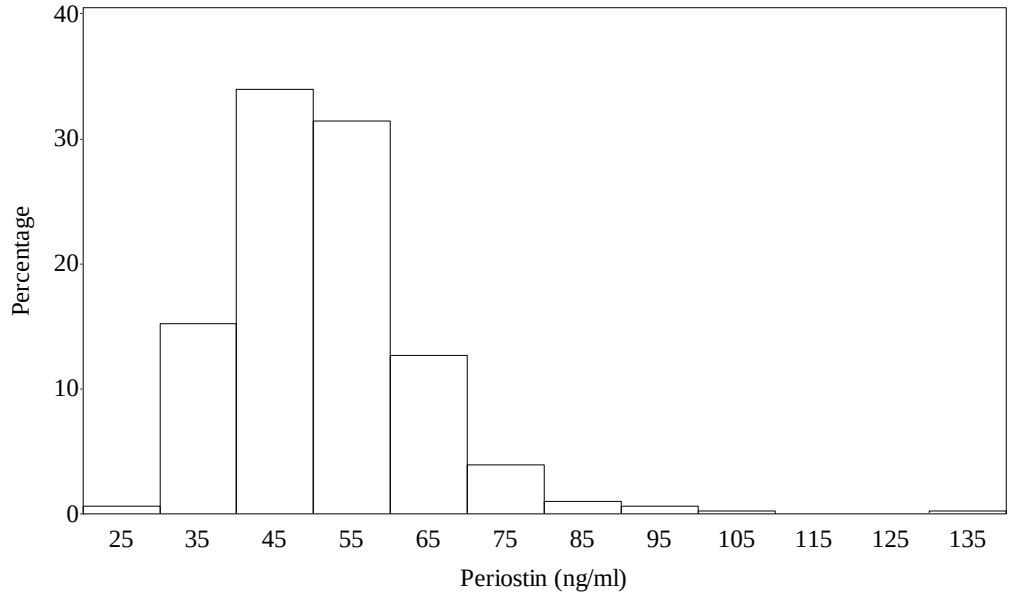
Variable (Yes, unless indicated)	**Number/480 (%)**
Sex Female	240 (50)
Ethnicity:	
European	420 (87.5)
Maori	19 (4.0)
Pacific	5 (1.0)
Asian	34 (7.1)
Other	2 (0.4)
Smoking:	
Ever	168 (35)
Current	22 (4.6)
Atopy:	
Eczema	87 (18.1)
Hayfever	196 (40.8)
Nasal polyps	13 (2.7)
Cardiovascular:	
Heart failure	1 (0.2)
Hypertension	85 (17.7)
Myocardial infarction	11 (2.3)
Valve disease	13 (2.7)
Metabolic/endocrine:	
Diabetes (type I and type II)	15 (3.1)
Post-menopausal	90 (18.8)
Hypercholesterolemia	99 (20.6)
Osteoarthritis	50 (10.4)
Osteoporosis	8 (1.7)
Inflammatory:	
Inflammatory bowel disease	5 (1.0)
Inflammatory arthritis	28 (5.8)
Psoriasis	27 (5.6)
Other:	
Exercise*	213 (44.4)
Gastro-oesophageal reflux disease	74 (15.4)
Dental caries	20 (4.2)

*Exercise: a positive response to the question ‘In the last seven days, have you exercised for >30 minutes at a time and had to breathe hard/ it would have been difficult to hold a conversation?’

Distribution of serum periostin

The distribution of serum periostin is shown in Figure 3a. The mean (standard deviation (SD)) periostin level was 51.2 (11.9) ng/ml, median (IQR) was 50.1 (43.1 to 56.9) ng/ml, and the minimum and maximum levels were 28.1 and 136.4 ng/ml, respectively. There was one outlying periostin value of 136.4 ng/ml in a 74 year old male with a history of nasal polyps, and highest serum IgE of all participants (2608 IU/L), FeNO (79 ppb) above the 95th percentile for all participants, an FEV1 of 75.8%predicted and an FEV1/FVC ratio of 75%.

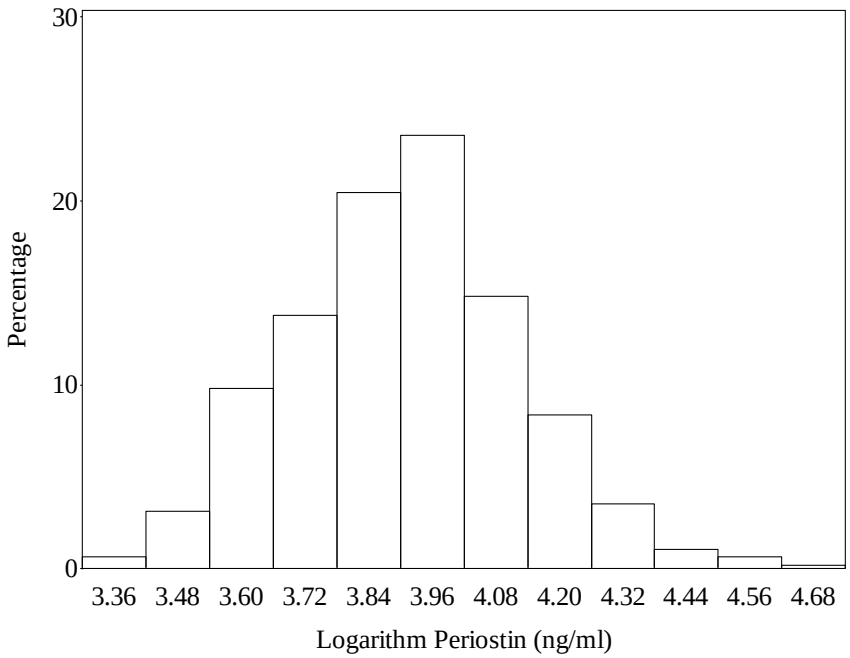
Figure 4.2: Frequency histograms of serum periostin levels



Formal tests for normal distribution of serum periostin and logarithm periostin

The four tests for normal distribution of serum periostin provided strong evidence of non-normality (Shapiro-Wilk P<0.001, Kolmogorov-Smirnov P<0.01, Cramer-von Mises P<0.005 and Anderson-Darling P<0.005). With the outlying value of serum periostin retained in the dataset, the tests for the logarithm transformed periostin (log periostin) were statistically significant for only the Shapiro-Wilk test (P=0.009; P>0.15 for the other tests). All four tests were not statistically significant with the outlying value removed (P=0.57 for Shapiro-Wilk and P>0.15 for the other tests), indicating that normality assumptions for the distribution of periostin are well met on the log transformed scale with the outlying value removed (Figure 4.3).

Figure 4.3: Frequency histogram for logarithm transformed serum periostin levels with outlying value (136.4 ng/ml) removed from dataset



Associations with sex and age

There was no evidence of an association between log periostin and sex (Table 2, Figure 4.4) or log periostin and age (Table 2, Figure 4.5), with the one outlying data point removed from the dataset.

Reference range based on 90% Confidence interval for an individual predicted value

Based on the model without age or sex as explanatory variables and removing the one outlying value, the log periostin was 3.91 (90% CI 3.55 to 4.26), and exponent was 49.9 (90% CI 35.0 to 71.1), resulting in 90% confidence intervals of 35.0 ng/ml and 71.1 ng/ml. The corresponding 95% confidence intervals were 32.7 and 76.1 ng/ml.

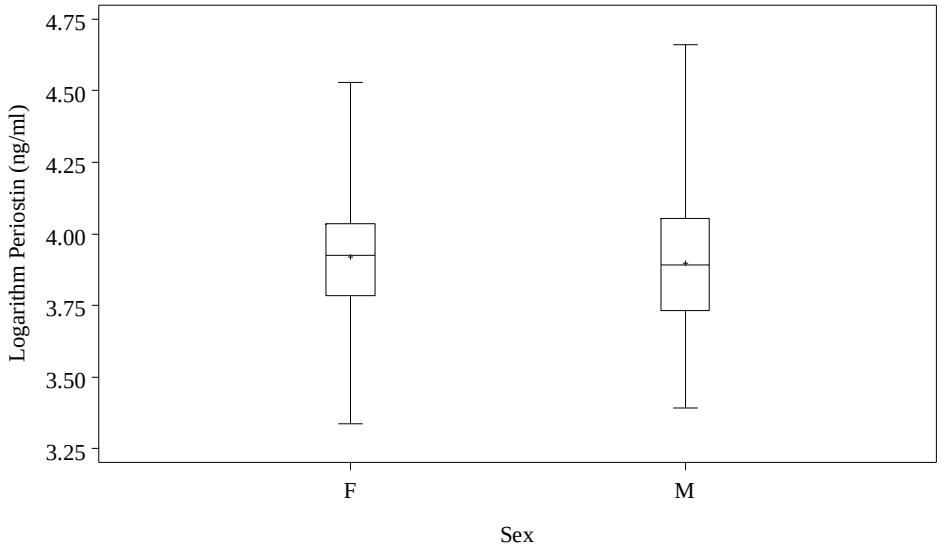
Table 4.2: Serum periostin levels by sex and age #

Variable	Mean (SD)	Median (IQR)	Min to Max
Periostin:			
Male N=239	50.6 (11.8)	49.0 (41.8 to 57.6)	29.8 to 105.7
Female N=240	51.5 (10.8)	50.7 (44.0 to 56.5)	28.1 to 92.8
Logarithm periostin:			
Male N=239	3.90 (0.22)	3.89 (3.73 to 4.05)	3.39 to 4.66
Female N=240	3.92 (0.21)	3.93 (3.78 to 4.03)	3.34 to 4.53

Analysis variable	Estimate (95% CI)	P
Logarithm periostin: Female minus Male	0.021 (-0.017 to 0.06)	0.28
Exponent of logarithm difference: ratio of mean periostin Female to Male	1.02 (0.98 to 1.06)	
Coefficients for age		
Change in logarithm periostin per year older	0.00013 (-0.00083 to 0.0011)	0.82
Change in logarithm periostin per decade older	0.00129 (-0.00831 to 0.0109)	
Exponent of change in logarithm periostin: Ratio of mean periostin per year older	1.000 (0.999 to 1.001)	
Exponent of change in logarithm periostin: Ratio of mean periostin per decade older	1.001 (0.991 to 1.011)	

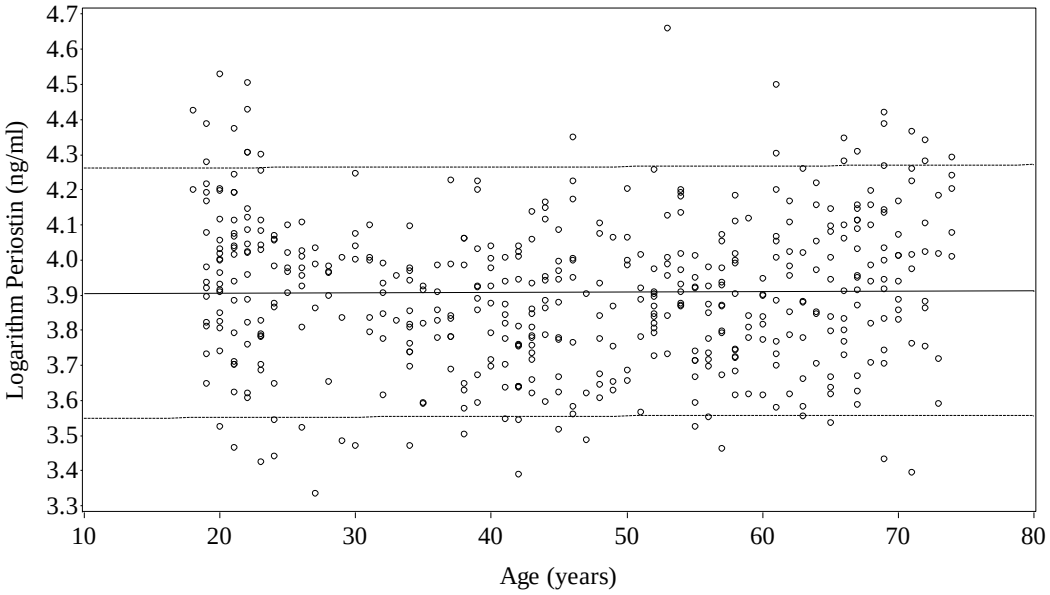
Outlying periostin value removed from the dataset

Figure 4.4: Box plot of logarithm transformed serum periostin levels in female (F) and male (M) individuals, with outlying value removed from dataset



The horizontal lines are the 25th, median, and 75th percentiles, the symbol is the mean, and the whiskers extend from the minimum to maximum value.

Figure 4.5: Scatter plot of logarithm transformed serum periostin levels versus age with linear regression line and 90% confidence limits for individual predictions, with outlying value removed from dataset



Associations between serum periostin and clinical, lung function and Th2 biomarker variables

(i) Continuous variables:

The association between log periostin and the key variables are shown in Table 3 and Figures 4.6 – 4.13. IgE and FeNO had highly skew distributions and these variables were also log transformed. There were weak positive associations between log periostin and blood eosinophil count and log IgE, but not log FeNO. There was a weak negative association between log periostin and FEV_1, FVC, BMI and creatinine clearance.

Table 4.3: Association between logarithm periostin and continuous variables

Variable	Difference in logarithm periostin per unit change (95% CI)	Ratio of mean periostin per unit change (95% CI)	R-squared (%)	r	P
Clinical:					
BMI (kg/m^2)	-0.0089 (-0.013 to -0.0048)	0.991 (0.987 to 0.995)	3.7	-0.19	**<0.001**
Creatinine (μmol/L)	-0.0012 (-0.0026 to 0.0003)	0.999 (0.997 to 1.000)	0.5	-0.07	0.113
Creatinine clearance (ml/min)	-0.0009 (-0.0016 to -0.0003)	0.999 (0.998 to 1.000)	1.7	-0.13	**0.004**
Urea (mmol/L)	-0.0004 (-0.0187 to 0.0107)	0.996 (0.981 to 1.011)	0.01	-0.02	0.596
Haemoglobin (g/L)	-0.0018 (-0.0032 to -0.0009)	0.998 (0.997 to 1.00)	1.2	-0.11	**0.015**
White cell count (x10^9/L)	-0.0034 (-0.0173 to 0.0106)	0.997 (0.983 to 1.011	0.01	-0.02	0.635
Lymphocyte count (x10^9/L)	0.0127 (-0.0235 to 0.049)	1.013 (0.977 to 1.05)	0.1	0.03	0.491
Pack years	0.0003 (-0.0024 to 0.0029)	1.00 (0.998 to 1.003)	0.01	0.01	0.842
Years smoking	-0.0008 (-0.0029 to 0.0013)	0.999 (0.997 to 1.001)	0.01	-0.03	0.464
Lung function:					
FEV_1 (L)	-0.0287 (-0.0501 to -0.0074)	0.97 (0.951 to 0.993)	1.4	-0.12	**0.009**
FEV_1/FVC	0.0341 (-0.2361 to 0.3043)	1.04 (0.79 to 1.36)	0.01	0.01	0.804
FVC (L)	-0.0259 (-0.0432 to -0.0086)	0.974 (0.958 to 0.991)	1.8	-0.13	**0.003**
FEV_1 % predicted	-0.0016 (-0.0031 to -0.0001)	0.998 (0.997 to 1.00)	0.9	-0.09	**0.041**
FVC % predicted	-0.0015 (-0.0030 to 0)	0.998 (0.997 to 1.0)	0.8	-0.09	0.053
Th2 markers:					
Eosinophil (x10^9/L)	0.19 (0.016 to 0.36)	1.20 (1.02 to 1.43)	1.0	0.10	**0.032**
FeNO (ppb)	0.0009 (-0.0001 to 0.0019)	1.00 (1.00 to 1.002)	0.7	0.08	0.067
Log FeNO	0.0257 (-0.0093 to 0.0608)	1.03 (0.991 to 1.063)	0.4	0.07	0.150
Log IgE	0.0234 (0.0109 to 0.0359)	1.024 (1.011 to 1.037)	2.8	0.17	**<0.001**

Figure 4.6: Scatterplot of association between logarithm transformed serum periostin levels and BMI

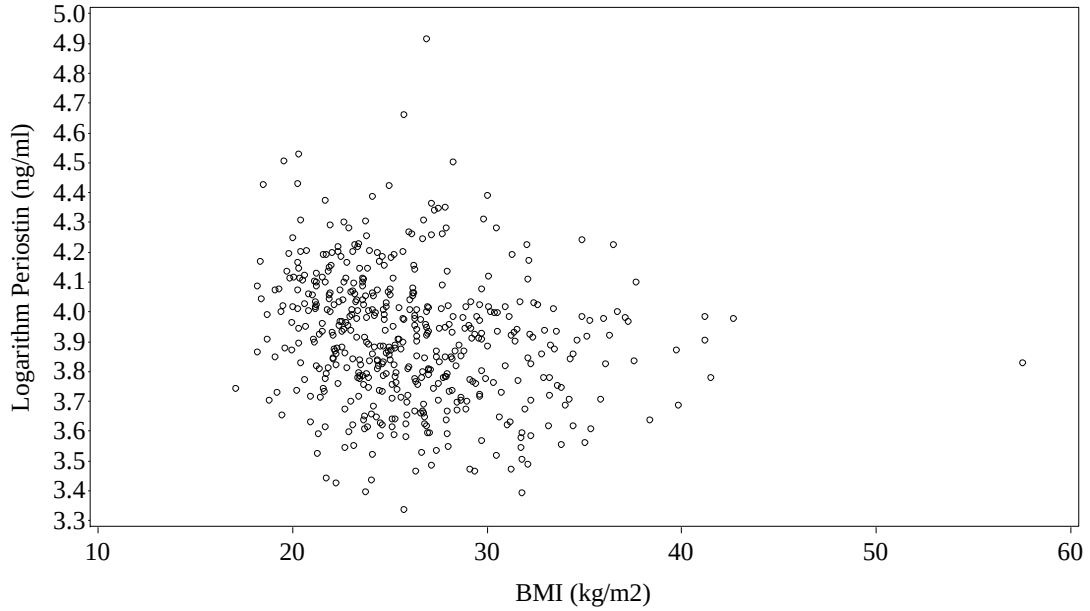


Figure 4.7: Scatterplot of association between logarithm transformed serum periostin levels and Serum Creatinine

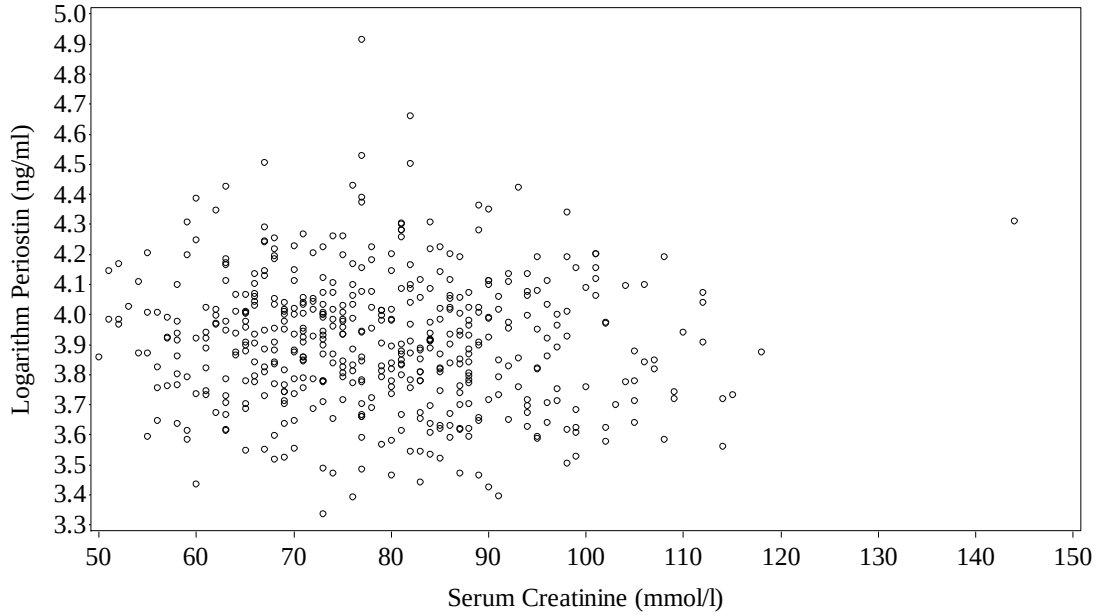


Figure 4.8: Scatterplot of association between logarithm transformed serum periostin levels and estimated eGFR

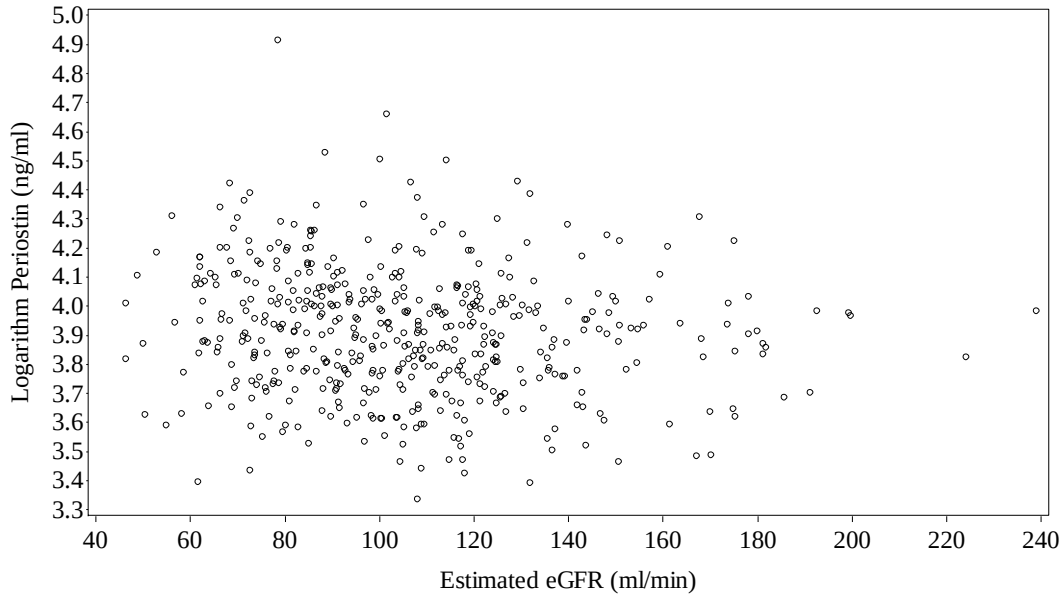


Figure 4.9: Scatterplot of association between logarithm transformed serum periostin levels and FEV1%

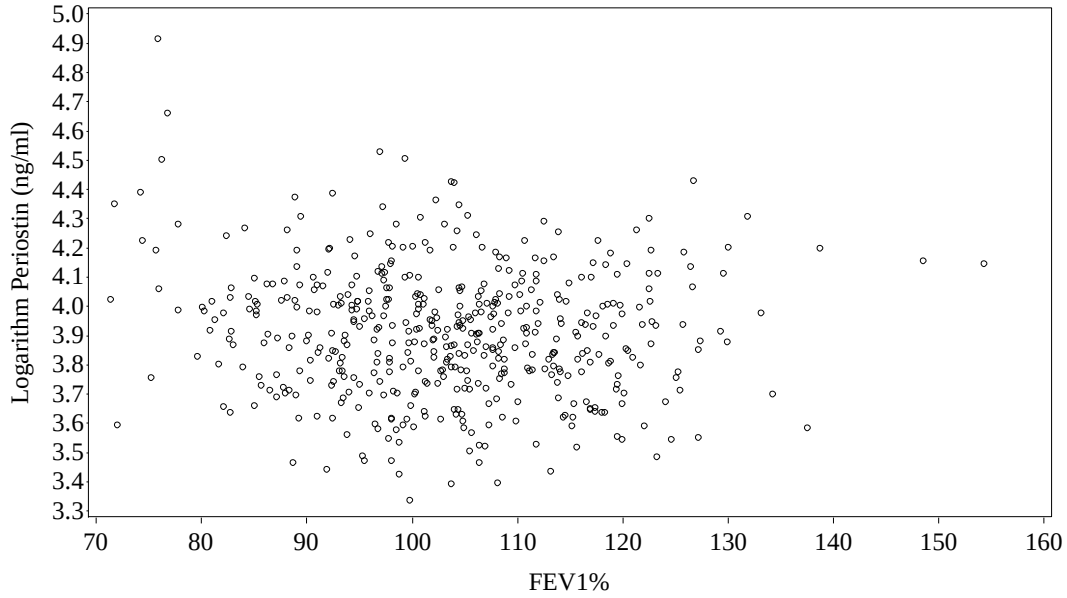


Figure 4.10: Scatterplot of association between logarithm transformed serum periostin levels and FVC%

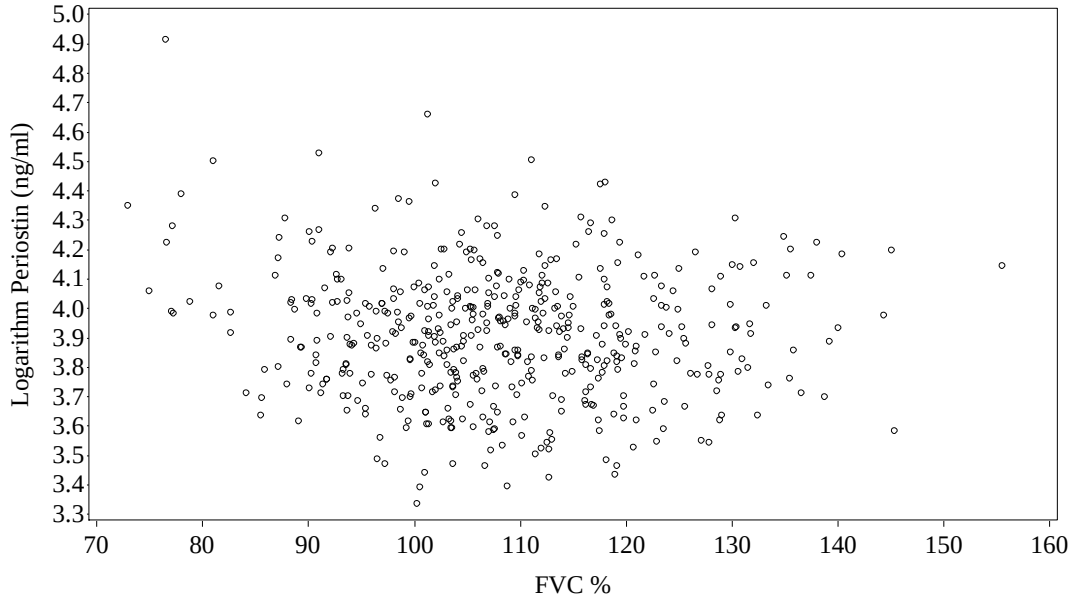


Figure 4.11: Scatterplot of association between logarithm transformed serum periostin levels and Eosinophil count

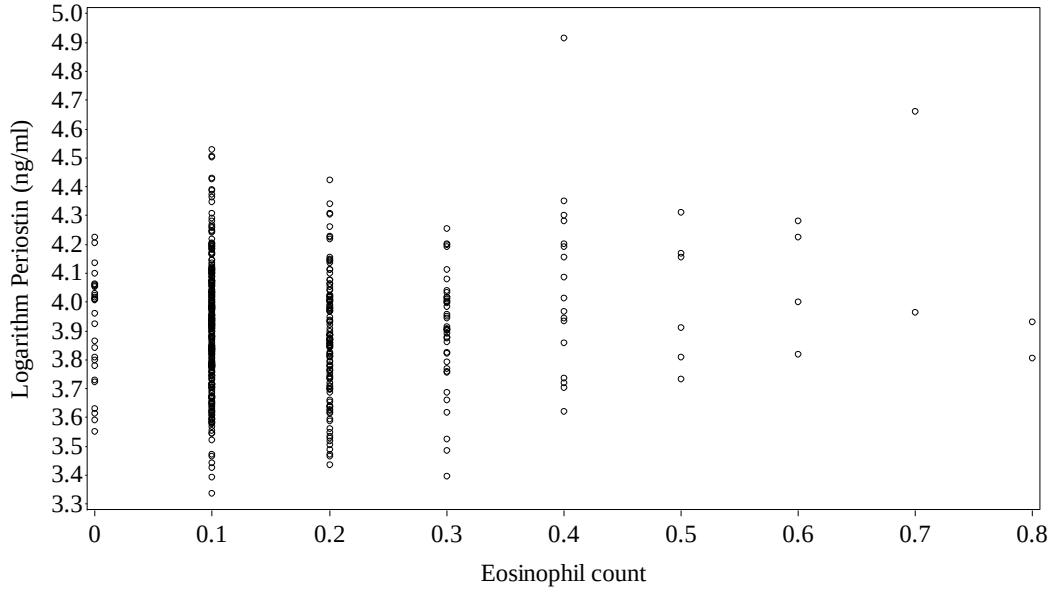


Figure 4.12: Scatterplot of association between logarithm transformed serum periostin levels and logarithm FeNO

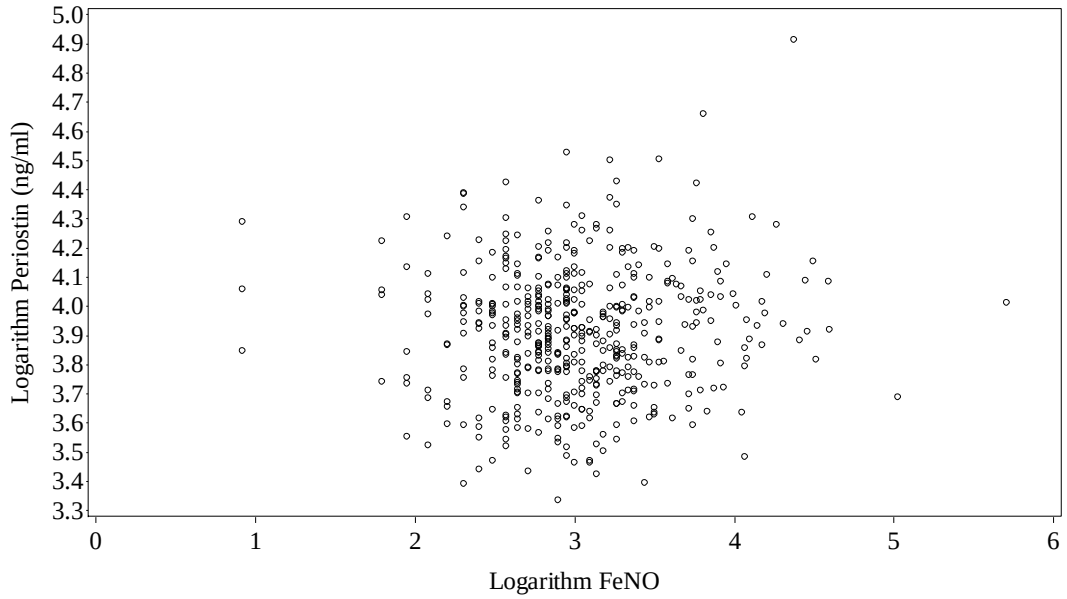
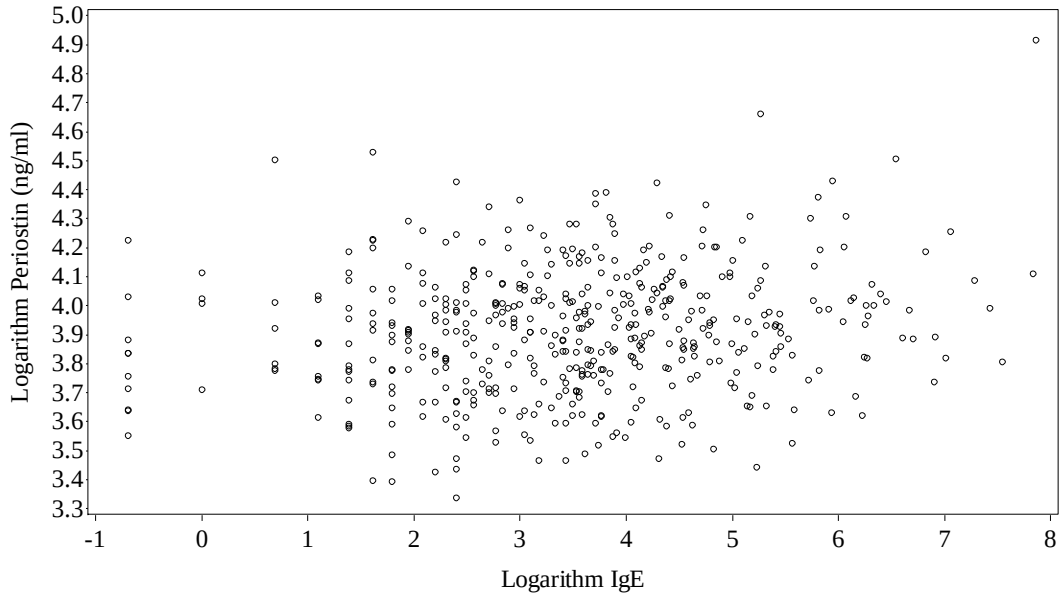


Figure 4.13: Scatterplot of association between logarithm transformed serum periostin levels and logarithm IgE



(ii) Categorical variables:

The serum periostin levels in groups defined by categorical variables are shown in Table 4.4. Periostin levels were significantly lower in current smokers, and individuals with cardiac valve disease (Table 4.5).

Table 4.4: Serum periostin levels in groups defined by categorical variables

Variable	Mean (SD) (ng/ml)	Median (IQR) (ng/ml)	Min to Max (ng/ml)
Ethnicity:			
European N=420	50.9 (12.1)	49.7 (42.8 to 56.5)	28.1 to 136.4
Maori N=19	51.3 (9.6)	50.7 (44.1 to 56.4)	40.0 to 80.6
Pacific N=5	53.5 (6.2)	53.5 (48.8 to 54.6)	47.4 to 63.2
Asian N=34	55.2 (11.0)	55.5 (48.2 to 63.4)	30.7 to 77.5
Other N=2	48.2 (7.5)		42.9 to 53.5
Smoking:			
Ever			
Yes N=168	51.1 (11.8)	50.3 (42.8 to 55.9)	29.7 to 105.7
N=312	51.3 (12.0)	49.9 (43.6 to 57.6)	28.1 to 136.4
Current			
Yes N=22	46.1 (10.5)	43.3 (38.6 to 54.6)	29.7 to 68.3
No N=458	51.5 (12.0)	50.3 (43.6 to 57.1)	28.1 to 136.4
Atopy:			
Eczema			
Yes N=87	51.6 (11.6)	50.7 (42.0 to 59.6)	31.3 to 90.6
No N=393	51.1 (12.0)	49.9 (43.3 to 56.5)	28.1 to 136.4
Hayfever			
Yes N=196	50.5 (11.1)	49.0 (42.3 to 55.7)	31.3 to 105.7
No N=284	51.7 (12.5)	50.6 (43.7 to 57.9)	28.1 to 136.4
Nasal polyps			
Yes N=13	57.4 (26.5)	51.6 (41.7 to 63.9)	33.7 to 136.4
No N=467	51.0 (11.3)	50.0 (43.3 to 56.8)	28.1 to 105.7
Cardiovascular:			
Heart failure			
Yes N=1	76.8		
No N=479	51.2 (11.9)	50.0 (43.1 to 56.9)	28.1 to 136.4
Hypertension			
Yes N=85	52.6 (12.1)	51.7 (42.7 to 58.9)	33.9 to 90.1
No N=395	50.9 (11.9)	49.8 (43.5 to 56.5)	28.1 to 136.4
Myocardial infarction			
Yes N=11	50.3 (11.5)	51.6 (38.9 to 55.9)	34.3 to 72.5
No N=469	51.2 (12.0)	50.0 (43.2 to 56.9)	28.1 to 136.4
Valve disease			
Yes N=13	60.5 (14.3)	59.2 (51.2 to 66.7)	37.6 to 92.8
No N=467	51.0 (11.8)	49.9 (42.9 to 56.5)	28.1 to 136.4

Metabolic/endocrine:			
Diabetes			
Yes N=15	52.2 (11.9)	49.6 (43.8 to 55.6)	37.2 to 80.6
No N=465	51.2 (12.0)	50.2 (43.1 to 56.9)	28.1 to 136.4
Post-menopausal			
Yes N=90	51.8 (10.5)	50.3 (44.1 to 56.6)	31.0 to 80.6
No N=150	51.2 (11.0)	50.7 (43.9 to 56.5)	28.1 to 92.8
Hypercholesterolemia			
Yes N=99	50.0 (10.5)	49.3 (41.9 to 55.3)	29.9 to 80.6
No N=381	51.5 (12.3)	50.4 (43.6 to 57.7)	28.1 to 136.4
Osteoarthritis			
Yes N=50	53.3 (16.2)	50.2 (44.6 to 55.9)	29.9 to 136.4
No N=430	51.0 (11.3)	50.1 (42.9 to 56.9)	28.1 to 105.7
Osteoporosis			
Yes N=8	55.7 (12.3)	52.1 (48.9 to 64.6)	37.7 to 76.8
No N=472	51.1 (11.9)	49.9 (43.1 to 56.9)	28.1 to 136.4
Inflammatory:			
Inflammatory bowel disease			
Yes N=5	58.4 (7.8)	60.3 (52.2 to 65.5)	48.5 to 65.6
No N=475	51.1 (12.0)	49.9 (43.1 to 56.9)	28.1 to 136.4
Inflammatory arthritis			
Yes N=28	51.5 (10.5)	49.5 (42.1 to 60.6)	36.4 to 76.8
No N=352	51.2 (12.0)	50.1 (43.3 to 26.8)	28.1 to 136.4
Psoriasis			
Yes N=27	52.7 (13.3)	50.7 (43.5 to 58.3)	33.3 to 90.1
No N=453	51.1 (11.9)	50.0 (43.1 to 56.9)	28.1 to 136.4
Other:			
Exercise			
Yes N=213	51.5 (11.7)	50.4 (43.8 to 58.6)	28.1 to 92.8
No N=267	51.0 (12.1)	49.5 (42.9 to 55.8)	29.7 to 136.4
Gastro-oesophageal reflux disease			
Yes N=74	51.5 (11.6)	50.3 (42.8 to 57.6)	32.7 to 80.6
No N=406	51.2 (12.0)	50.1 (43.3 to 56.9)	28.1 to 136.4
Dental caries			
Yes N=20	51.3 (12.4)	53.1 (41.6 to 57.9)	29.7 to 80.6
No N=460	51.2 (11.9)	49.9 (43.3 to 56.9)	28.1 to 136.4

Table 4.5: Summary of general linear model (t-tests for dichotomous variables and ANOVA for Ethnicity) using logarithm transformed periostin

Variable	Difference in logarithm periostin Yes minus No (95% CI)	Ratio of mean periostin Yes compared to No (95% CI)	R-squared (%)	P
Ethnicity:[1]			1.1	0.14
Maori vs European	0.0183 (-0.0828 to 0.1194)	1.018 (0.921 to 1.127)		
Pacific vs European	0.0703 (-0.1236 to 0.2642)	1.073 (0.884 to 1.302)		
Asian vs European	0.0875 (0.0106 to 0.1643)	1.091 (1.011 to 1.179)		
Smoking:				
Ever	-0.0045 (-0.0458 to 0.0369)	0.996 (0.955 to 1.038)	0.001	0.83
Current	-0.1088 (-0.2026 to -0.0151)	0.897 (0.817 to 0.985)	1.1	**0.023**
Atopy:				
Eczema	0.0096 (-0.0415 to 0.0608)	1.01 (0.959 to 1.063)	0.07	0.71
Hayfever	-0.0209 (-0.0609 to 0.0192)	0.979 (0.941 to 1.019)	0.2	0.31
Nasal polyps	0.0687 (-0.0526 to 0.1900)	1.071 (0.949 to 1.209)	0.3	0.27
Cardiovascular:				
Heart failure	NA	NA	NA	NA
Hypertension	0.0331 (-0.0185 to 0.0846)	1.034 (0.982 to 1.088)	0.3	0.21
Myocardial infarction	-0.0167 (-0.1484 to 0.1151)	0.983 (0.862 to 1.122)	0.01	0.80
Valve disease	0.1699 (0.0494 to 0.2904)	1.091 (1.011 to 1.179)	1.6	**0.006**
Metabolic/endocrine:				
Diabetes	0.0277 (-0.0764 to 0.1679)	1.023 (0.913 to 1.146)	0.03	0.69
Post-menopausal (women only)	0.0126 (-0.0413 to 0.0666)	1.013 (0.960 to 1.069)	0.10	0.65
Hypercholesterolemia	-0.0243 (-0.073 to 0.0244)	0.976 (0.93 to 1.025)	0.2	0.33
Osteoarthritis	0.0344 (-0.0301 to 0.0988)	1.035 (0.970 to 1.104)	0.2	0.30
Osteoporosis	0.0891 (-0.0647 to 0.2429)	1.093 (0.937 to 1.275)	0.3	0.26

Inflammatory:				
Inflammatory bowel disease	0.1507 (-0.043 to 0.3444)	1.163 (0.958 to 1.411)	0.5	0.13
Inflammatory arthritis	0.0112 (-0.0729 to 0.0953)	1.011 (0.930 to 1.10)	0.01	0.79
Psoriasis	0.0255 (-0.060 to 0.111)	1.026 (0.942 to 1.117)	0.1	0.56
Other:				
Exercise	0.0103 (-0.0294 to 0.0499)	1.007 (0.953 to 1.063)	0.05	0.61
Dental caries	0.0032 (-0.1019 to 0.0954)	0.997 (0.903 to 1.10)	0.02	0.95
Gastro-oesophageal reflux disease	0.0069 (-0.0477 to 0.0615)	1.007 (0.953 to 1.063)	0.01	0.80

[1] Note overall P for Ethnicity not statistically significant, comparisons for completeness only, 'Other', N=2, omitted

In t-tests and ANOVA differences the exponent of the difference in logarithm between categories is equivalent to the ratio of mean values of different categories.

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DISCUSSION

This study presents the distribution of serum periostin values in an adult population without asthma or COPD. There was no association between log periostin level and either sex or age. Normal reference range values are provided across the age range of 18 to 75 years, independent of sex, with 90% confidence intervals of 35.0 and 71.1 ng/ml respectively. We also identified that log periostin shows a modest positive association with the blood eosinophil count and log IgE, but not log FeNO, suggesting that it may measure related but different components of the Th2 inflammatory processes.

Main findings

It was observed that the distribution of serum periostin levels in this population without asthma or COPD was relatively wide, with a five-fold range of levels (28.1 to 136.4 ng/ml), and a marked right skew distribution. The median (IQR) serum periostin level of 50.1 ng/ml (43.1 to 56.9 ng/ml) was similar to the median (IQR) level of 54.0 ng/ml (45.1 to 65.6 ng/ml) reported by MRINZ in a random adult population with symptoms of obstructive airways disease, using the same laboratory and assay to measure serum periostin levels (Fingleton et al. 2015; Fingleton et al. submitted) These values are also similar to the median level of 50.2 ng/ml previously reported in adults with moderate and severe asthma inadequately controlled despite ICS therapy (Corren et al. 2011). Likewise, the mean serum periostin level of 51.2 ng/ml was similar to the mean value of 53 ng/ml reported in severe persistent allergic asthma uncontrolled despite treatment with high-dose ICS plus long-acting beta agonist therapy (Hanania et al 2013), and 57 ng/ml reported in the New Zealand respiratory health survey of a random adult population with symptoms of obstructive airways disease (Fingleton et al. submitted) While recognizing the modest reduction in serum periostin levels with ICS therapy (Fingleton et al. submitted), these findings suggest that periostin is not a measure which can usefully discriminate patients with asthma receiving different treatment regimens across a range of severity, or from a population without asthma or COPD. It also suggests that asthma is not the major determinant of serum periostin levels and those pathophysiological processes related to its function as a matricellular protein, with

expression typically confined to cells of mesenchymal origin, are likely to play a greater role (Conway et al. 2014).

To enhance the accuracy of serum periostin in guiding personalized asthma management such as identifying responders to monoclonal antibody therapy directed against IL-13 (Corren et al. 2011) and IgE (Hanania et al 2013) physicians need to be able to interpret periostin values in the context of an individual patient's characteristics, risk factors, and medical history. The findings of this study confirm those from a population with asthma and COPD (Fingleton et al. submitted), that periostin values do not need to be adjusted to take account of a patient's age, sex, or common comorbidities. The finding of lower periostin levels in current smokers, but not ever smokers, may relate to the suppressive effects of smoking on the number of blood eosinophils (Van der Vaart et al. 2004) and is consistent with previous observations of reduced FeNO in current smokers (Travers et al. 2007). The observation that the small number of individuals with a history of cardiac valve disease had higher periostin levels is of interest as periostin plays a role in heart valve development, remodelling and valvular disease (Conway et al. 2011), and its expression in cardiac tissues is up-regulated after cardiac injury (Dorn II 2007; Kuhn et al. 2007). There was otherwise no association between serum periostin and an individual's history of ischemic heart disease, or hypertension: there were insufficient subjects with heart failure to examine this association. This study was unable to confirm the previous finding in a population with symptoms of airflow obstruction of a negative association with the presence of gastro oesophageal reflux disease (GORD) (Fingleton et al. submitted). However, a weak inverse correlation between BMI and serum periostin was confirmed, indicating that periostin may be preferentially deposited in rather than remaining in the serum (Bolton et al. 2009; Lu et al. 2014; Wu et al. 2014). Serum periostin was also negatively associated with creatinine clearance, but not serum creatinine or urea. This is consistent with previous reports that periostin is a mediator and marker of chronic kidney disease and acute kidney injury (Mael-Ainin et al. 2014; Satirapoj et al. 2012). Further study of the effect of acute and chronic renal impairment on serum periostin levels is indicated.

In terms of Th2 biomarkers, the associations between serum periostin and blood eosinophil count and log IgE were statistically significant albeit weak, and there was no association with log FeNO. Furthermore, periostin levels were not raised in individuals with comorbidities defined by atopic status, such as hayfever or eczema although these were self-reported and were not categorized by severity, treatment or current symptoms. These findings complement our findings in the population with symptoms of airflow obstruction of modest associations between serum periostin and blood eosinophil counts, IgE and FeNO, and higher periostin levels in atopy defined by Phadiatop (Conway et al. 2014).

They also correlate with the findings in a study of 224 patients with asthma where a weak but positive relationship between periostin and serum IgE and blood eosinophil counts were seen (Matsumoto et al. 2014). Together they are consistent with the observation that FeNO and blood eosinophil counts are not clearly related (Malinovschi et al 2013; Pavord et al. 2013) and suggest that these Th2 biomarkers might identify different aspects of Th2 mediated inflammation.

The general population studied was predominantly New Zealand European (87.5%), and thus our results are likely to be generalizable to Caucasian populations. We found a trend towards higher serum periostin levels in participants self-identifying as Asian, consistent with our previous observation that the mean serum periostin in adults with symptomatic airflow obstruction was higher in Asian compared with NZ European ethnicity (69.6 ng/ml vs 56.9 ng/ml respectively) (Fingleton et al. submitted). The reference range of serum periostin in different populations is not known and should be a focus for future research.

Methodological Issues

The recommendations for establishing health-associated reference intervals for a quantitative clinical laboratory tests were closely followed (Horowitz et al. 2008). Reference intervals for adults between 18 and 75 years of age were developed to enable generalisability to the age range of the population in whom monoclonal antibody therapy is currently trailed in severe asthma (Corren et al. 2011). Subjects were excluded if they had a diagnosis of asthma or COPD, current symptoms suggestive of airflow obstruction, or a recent respiratory tract infection (within the last three weeks). Participants were also excluded if they had major surgery, dental procedures or a fracture within the last three months due to the known bone and connective tissue origin of periostin (Kruzynska-Frejtag et al. 2004; Oku et al. 2008; Takeshita et al. 1993), and its role in extracellular matrix deposition, fibrosis, tissue remodelling and repair (Conway et al. 2014; Gordon et al. 2012; Hamilton 2008; Takayama et al. 2006). The effect of these factors on serum periostin levels will need to be determined.

In addition to these exclusion criteria, partitioning criteria were employed to divide the reference sample into significant sub-classes based on age and sex. The study was powered to ensure that the recommended minimum of 120 reference subjects was included in each age band (Horowitz et al. 2008). The width of each confidence interval depends on both the number of reference subjects and the distribution of the observed reference values. As the distribution of serum periostin was right skewed the values were logarithm transformed to ensure a Gaussian distribution. There was a 74 year old male with a history of nasal polyps, who was highly atopic and was not included in the calculation of

the reference ranges due to an outlying periostin level of 136.4ng/ml. As recommended (Horowitz et al. 2008), direct sampling techniques were used in which reference individuals were selected from a reference population using specific criteria, applied a priori, before the samples were collected and analysed. Furthermore, all analytical factors including sample collection, handling, storing and transfer of the blood samples were carefully defined, with periostin measurements undertaken in a reference laboratory.

Some of the associations we have found should be interpreted cautiously. In this exploratory study we did not adjust for performing multiple statistical tests and so some of our associations may be due to Type I error rate inflation. Regardless, all the statistically significant associations had weak exploratory power, with a highest univariate R-squared of 3.7%. In addition, some of the non-significant differences in periostin levels between subgroups defined by disease category may have been due to an insufficient number of individuals with uncommon comorbidities, such as inflammatory bowel disease and nasal polyps, resulting in a Type II error.

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CONCLUSION

The reference range values of serum periostin levels in a mainly Caucasian population without asthma or COPD are presented. From these results it can be suggested that serum periostin levels do not need to be adjusted for age, sex or common comorbidities, although there are lower periostin values in current smokers. Further study is required in specific conditions identified in this study such as acute or chronic renal disease and valvular heart disease and additionally in patients with recent fractures, surgery, and dental procedures who were excluded from this study. The role of periostin in the pathogenesis of Th2-driven asthma, and as a predictor of treatment responsiveness in asthma, will also need to be confirmed by further investigation, with reference to the distribution of serum periostin levels determined in this study.

Part III

STUDY B

7

METHODS

This non-experimental cohort observational study recruited adults aged 18 to 75 years from the Medical Research Institute of New Zealand (MRINZ) volunteer databases, Capital Coast District Health Board employees and word of mouth. In total 32 participants were enrolled; 16 participants with a doctor diagnosis of asthma who were prescribed maintenance ICS and LABA therapy, and on a stable ICS dose for the last three months (asthma group), and 16 participants who neither had a doctor diagnosis of asthma, symptoms of wheeze, or inhaler use in the past 12 months (non-asthma group). Exclusion criteria for both groups included: chronic bronchitis or COPD, known pregnancy, active (current, or within the three weeks prior to the visit) upper or lower respiratory tract infection, any of the following within the last 3 months; hospital admission, major surgery requiring general anaesthetic, dental extractions or root canal procedures and bone fracture and finally, any significant comorbidities or any safety concerns at the investigator's discretion.

Ethical approval was given by the Central Regional Ethics Committee of New Zealand (13/NTB/184/AM02). The trial was prospectively registered with Australia New Zealand Trials Registry (ACTRN12614000072617) and written informed consent was obtained from all participants prior to testing.

STUDY PROCEDURES

Participants attended the MRINZ outpatient facility at Capital and Coast District Health Board (CCDHB Wellington Hospital) for an initial visit (Visit 1) where the following procedures were completed: written informed consent, completion of the General Health Questionnaire, measurement of Body Mass Index (BMI) and training in spirometry technique. Participants then attended the Clinical Trials Unit (CTU) at CCDHB, where long stay facilities were available, on a second occasion (Visit 2). Participants were scheduled at 15 minute intervals at the CTU from 6am through to 8am in order to allow study

investigators to complete procedures as per protocol in a timely fashion. During visit 2 blood samples were drawn every two hours for serum periostin levels and peripheral blood eosinophil counts. Following each blood sample measures of FeNO, forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were performed. Six measurements were taken over the 10-hour period. Participants with asthma also completed the Asthma Control Questionnaire-5 (ACQ-5) and the Asthma Quality of Life Questionnaire with Standardized Activities (AQLQ(S)) to provide more information about their current asthma control.

General Health Questionnaire:

The general health questionnaire was used to obtain the current health status of participants, including a history of cancer, cardiovascular disease, gastro-oesophageal reflux (GORD), rhinitis, sinusitis, and eczema (Appendix 1). The medical history was obtained using questions drawn from a series of validated questionnaires including the Health Assessment Questionnaire (HAQ) from Stanford University (Bruce et al. 2003) with co-morbidities recorded using questions from the American Thoracic Society (ATS) Division of Lung Diseases-78 (DLD-78) questionnaire. Following a literature review, questions were formulated to capture information about those body systems where periostin is known to be involved. This information was then used to exclude participants where there may be a confounding illness or injury such as recent dental extraction, or bone fracture. The questionnaire was administered by doctors and trained investigators only. All investigators were trained by the lead investigator to ensure consistency in the administration of the questionnaire. The justification for the general health questionnaire with references has been provided in Appendix 2.

Respiratory Health Questionnaires:

The AQLQ(S) is a validated 32 question self-administered questionnaire which was used to obtain an assessment of asthma severity in the two weeks prior to Visit 2 (Juniper et al. 1992).
The ACQ-5 is a validated five question self-administered questionnaire that was used to obtain severity of asthma in the one week prior to Visit 2 (Juniper et al. 2005). These questionnaires were administered to the asthma group only.

Lung function and FeNO:

Prior to baseline lung function testing participants avoided eating for one hour, smoking tobacco for two hours, and caffeine ingestion for six hours as recommended in the ATS criteria (Miller et al. 2005). Caffeine was also withheld throughout the duration of the study and participants were asked to eat only within the first hour following spirometry testing not in the hour prior to the following measurement. No

participants were current smokers although they were not actively excluded based on current smoking status. Participants were not tested within three weeks of an upper or lower respiratory tract infection as nitric oxide levels have been shown to be elevated during an acute infection but return to baseline within three weeks following the onset of symptoms (Kharitonov et al. 1995; ATS 2005). Expired nitric oxide testing was performed prior to spirometry as spirometric manoeuvers have been shown to significantly reduce FeNO levels initially (1 and 5 minutes post spirometry), returning to baseline values at one hour following spirometry (ATS 2005; Deykin et al. 1998; Silkoff et al. 1999) It is postulated that the drop in FeNO of up to 18% is as a result of bronchospasm induced by repeated spirometric efforts (Silkoff et al.1999).

Expired Nitric Oxide

Expired Nitric Oxide (FeNO) was determined according to ATS guidelines (ATS 2005) with a nitric oxide monitor (NiOX MINO, Aerocrine AB, Solna, Sweden). The testing was performed in a seated position and was explained prior to commencing. Verbal cues and positive encouragement was given throughout the testing. A single test was performed as per manufacturer guidelines and supporting research published following the ATS guidelines in 2005 (Alving et al. 2006; Khalili et al. 2007; Menzies et al. 2007)

Participants exhaled completely and then inhaled ambient air through a nitric oxide scrubber to total lung capacity. They then exhaled against an automatically adjusting resistance to achieve a constant exhalation flow rate of 50 ml/s +/- 10%. Resistance was adjusted automatically so that an upper airway pressure of 10-20 cm H_2O was maintained throughout exhalation, sufficient to close the velum and exclude nasal air. FeNO measurements were taken from a stable plateau in exhaled nitric oxide concentration during an exhalation. Exhalations where flow rate and plateau criteria are not met were deemed not acceptable for measurement. Repeated exhalations were performed a maximum of six times to obtain an acceptable measurement.

Spirometry

Spirometry was performed with measurement of FEV_1 and FVC using a Masterscreen Pneumo (Masterscreen Version 2.0, Carefusion, Leibnizstrasse Hoechberg, Germany) in accordance with American Thoracic Society (ATS) guidelines. All testing was performed in a seating position with nose clips on. Testing was explained to the participant prior to starting and positive encouragement and verbal cues were given throughout the testing.

Acceptability Criteria

Individual tests were acceptable if they were free from:

- Artefacts
- Cough during the first second of exhalation
- Glottis closure that influences the measurement
- Early termination or cut-off
- Effort that is not maximal throughout
- Leak
- Obstructed mouthpiece (e.g. tongue in front of mouthpiece)

They had good starts:

- Extrapolated volume <5% of FVC or 150ml, whichever is greater

They showed satisfactory exhalation:

- Duration of ≥ 6s or a plateau in the volume-time curve or if the subject could not or should not continue to exhale.

Repeatability Criteria

Testing was repeatable if:

- The two largest values of FVC were within 150ml of each other
- The two largest values of FEV1 were within 150ml of each other.

Testing continued until three acceptable manoeuvres had been completed or the subject had performed eight manoeuvres and could not or should not continue with testing. According to ATS guideline participants who were unable to produce reproducible flow volume loops (within 150ml variation in FEV1 and FVC) were not excluded from analysis. In subjects who were not able to produce three acceptable flow volume loops comments regarding technical acceptability of their testing were made. Predicted FEV1 and FVC values were calculated using the GLI criteria which adjust for age, sex, height, weight and ethnicity (Quanjer et al. 2012).

Bloods:

Full blood count and differential (Sysmex platform, Mundelein, USA) were performed immediately. Blood eosinophil counts were measured to one decimal place, expressed as 10^9/L. A further blood sample was centrifuged and serum aliquots stored at -80°C, prior to analysis of serum periostin. Serum periostin levels were determined using the clinical trial version of the Elecsys® Periostin immunoassay

(Roche Diagnostics, Penzberg, Germany) intended for use on the cobas e 601. The Elecsys® Periostin immunoassay is an automated assay immunoassay is an automated assay based on the sandwich principle and employs two monoclonal antibodies targeted to periostin. The antibodies have previous been described in Jia et al. A sandwich complex is formed between periostin, a biotinylated antibody and a ruthenylated antibody, and is captured on the surface of added streptavidin-coated microparticles. The amount of captured complex and therefore the periostin level in the sample is measured using electrochemiluminescence technology (Jia et al. 2012).

Medication Use:

Participants with asthma were advised to take their regular ICS and LABA treatment in the morning prior to attending the clinic on Visit 2, and not to take any medication during the 10-hour study period. Short-acting beta agonist (SABA) medication was made available throughout the duration of the study with its use recorded.

STUDY POWER

The clinically important difference in serum periostin is unknown. A sample size of 16 was chosen, with 80% power, alpha 5%, based on a paired t-test, to detect a paired difference of 0.75 standard deviations, for continuous variables, which constitutes in general terms a 'large' difference. This same sample size also has good precision for estimation of variance.

STATISTICAL METHODS

Simple data descriptions are shown for the variables by asthma status. Serum periostin and FeNO have skewed distributions and are better analysed on the natural logarithm transformed scale. The exponent of a difference in logarithms can be interpreted as a ratio of means.

Mixed linear models were used to compare time zero with subsequent measurement time by asthma groups. Simple unpaired t-tests were used to compare the baseline values for asthma versus non-asthma.

A post hoc analysis was undertaken to determine the proportion of adults with asthma that would change classification from 'high periostin' to 'low periostin', or from 'low periostin' to 'high periostin'

based on the 0800 and 1800 hour periostin levels, utilizing the proposed periostin cut point of 50 ng/ml, used to determine responsiveness to monoclonal antibody therapies (Corren et al. 2011; Hanania et al. 2013). Similarly the proportion of adults with asthma that would change classification from 'high FeNO' to 'low FeNO' or from 'low FeNO' to 'high FeNO' based on the 0800 and 1800 hour FeNO levels, utilizing the proposed cut point of 19.5 ppb (Hanania et al. 2013), used to determine responsiveness to monoclonal antibody therapy directed against IgE, was determined. A similar analysis was not undertaken for eosinophils as the proposed cut-point was 0.26 x 10^9/L, whereas eosinophil measurements for this study were only able to be measured to one decimal point due to lab reporting restraints.

SAS version 9.3 was used in all analyses.

8

RESULTS

Characteristics of participants

The flow of participants in the study is shown in Figure 8.1

Baseline characteristics are shown in Table 1. The participants with asthma had mild to moderate airflow obstruction with a mean FEV_1 % predicted of 90.5%, had partially controlled asthma with a mean ACQ-5 score of 1.3 (uncontrolled ACQ5 >1.5) , and were prescribed maintenance ICS/LABA inhaler therapy with the ICS component prescribed in the mean daily dose of 489 µg/day of fluticasone propionate (FP) or equivalent. The non-asthma participants were younger, had a lower BMI than the asthma participants, and a pre-bronchodilator FEV_1/FVC >0.70.

There was no statistically significant difference in periostin levels between the asthma and non-asthma groups at baseline (0800), with mean periostin of 53.5 ng/ml and 50.5 ng/ml respectively. The difference (95% CI) in logarithm periostin was 0.058 (-0.13 to 0.25), P=0.54. This is equivalent to a mean ratio of serum periostin levels in the asthma versus non-asthma group of 1.06 (0.88 to 1.28). Mean FeNO was 26.1 ppb in asthma compared to 22.3 ppb in non-asthma, difference (95% CI) in logarithm FeNO 0.16 (-0.25 to 0.56), P=0.43. This is equivalent to a mean ratio of FeNO levels in the asthma versus non-asthma group of 1.17 (0.78 to 1.76). There was weak evidence of a higher mean blood eosinophil count, 0.31 x 10^9/L in the asthma group, compared to 0.19 x 10^9/L in non-asthma, difference (95% CI), 0.11 (-0.005 to 0.23), P=0.061.

Figure 8.1: Flow of Subjects in Study

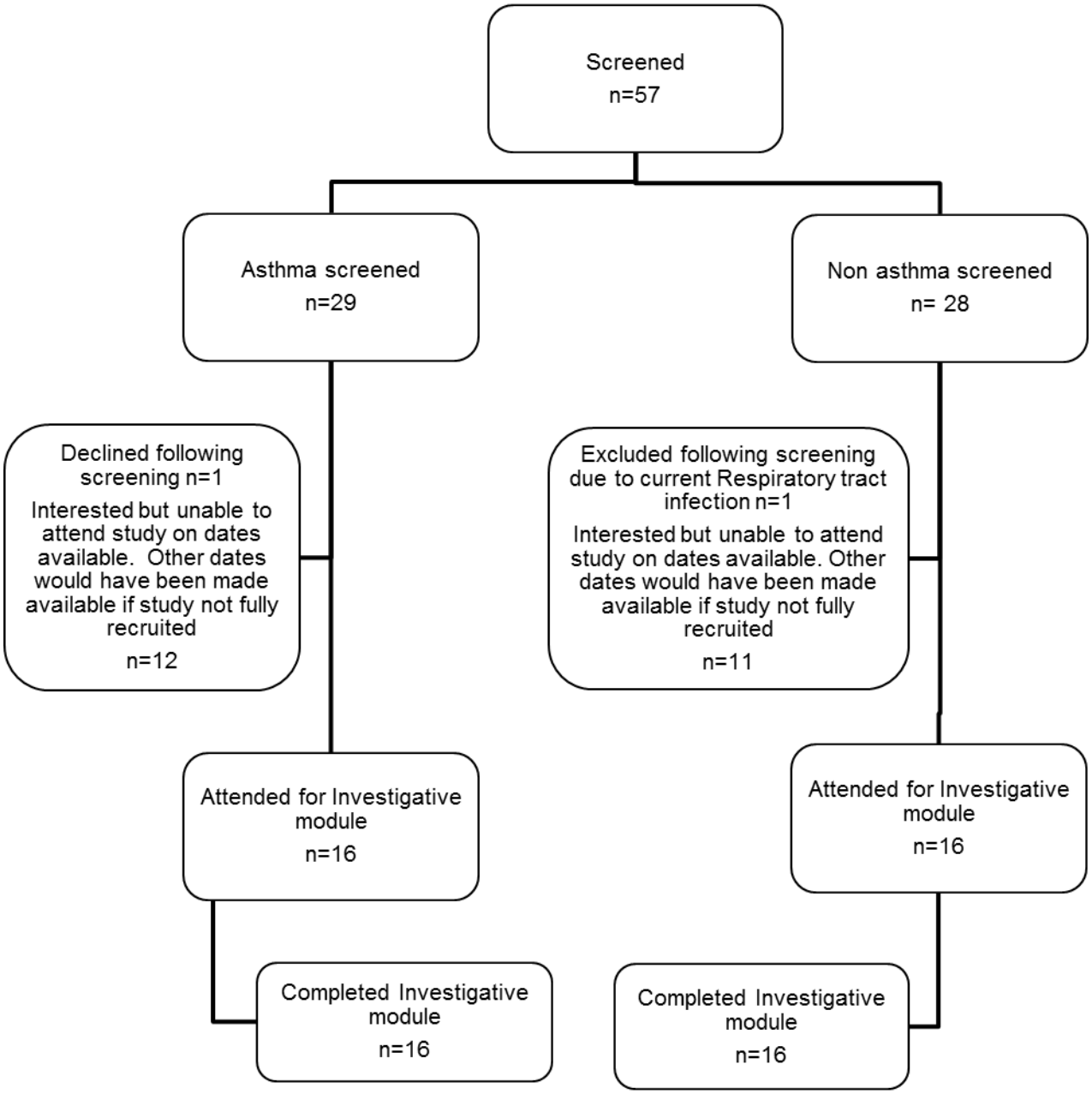


Table 8.1: Baseline characteristics of participants

Asthma

Variable N=16	Mean (SD)	Median (IQR)	Min to Max
Age (years)	45.4 (19.7)	47 (25 to 63.5)	19 to 73
BMI (kg/m^2)	32.2 (5.3)	33.4 (28.8 to 35.5)	20.9 to 42.2
FEV_1 % predicted#	90.5 (18.1)	96.6 (81.9 to 102.9)	50.6 to 111.6
ACQ-5¥	1.3 (1.2)	1.2 (0.4 to 1.4)	0 to 4.0
AQLQ(S)	5.71 (1.22)	6.05 (5.5 to 6.35)	2.8 to 7.0
Mean ICS dose‡	489 (105.3)	500 (500 to 500)	200 to 500
Eosinophil count (x 10^9/L)§	0.31 (0.20)	0.25 (0.20 to 0.35)	0 to 0.7
FeNO (ppb)	26.1 (13.3)	23.5 (16 to 36)	7 to 55
Periostin (ng/ml)	53.5 (13.6)	51.7 (41.5 to 63.7)	33.9 to 76.2

Non-Asthma

Variable N=16	Mean (SD)	Median (IQR)	Min to Max
Age (years)	28.1 (12.7)	23 (21 to 30.5)	20 to 68
BMI (kg/m^2)	23.8 (2.8)	22.8 (22.1 to 27.0)	19.7 to 28.3
FEV_1/FVC ratio†	0.85 (0.03)	0.86 (0.85 to 0.88)	0.77 to 0.88
FEV_1 % predicted#	97.9 (10.5)	96.3 (90.8 to 105.7)	80.5 to 120.9
Eosinophil count (x 10^9/L)	0.19 (0.11)	0.20 (0.10 to 0.25)	0.10 to 0.50
FeNO (ppm)	22.3 (14.1)	19.5 (15.5 to 24.5)	6.0 to 68.0
Periostin (ng/ml)	50.5 (13.0)	49.4 (42.5 to 62.7)	28.6 to 70.5

FEV_1 post-bronchodilator, expressed as % of normal predicted values

¥ ACQ-5: Asthma Control Questionnaire-5, a score >1.5 indicates uncontrolled asthma (Juniper et al. 2006)

‡ ICS daily dose expressed as fluticasone propionate equivalent (microgram/day)

§ Eosinophil laboratory reference range: 0.0 to 0.5 x 10^9/L, (measured in increments of 0.1)

† FEV_1/FVC ratio, pre-bronchodilator

Day-time changes in Asthma

The serum periostin level decreased from a mean of 53.5 ng/ml, at 0800 hours, to 50.9 ng/ml at 1800 hours. There was strong evidence, overall P <0.001, that the means by time were different, shown in Table 8.2, Figure 8.2, Table 8.3 and Figure 8.3. Compared with baseline, the log periostin was statistically significantly lower from the 4-hour (1200 hours) to the 10-hour (1800 hours) time points. The size of the difference remained constant from the 4-hour time period with an estimated difference in log periostin ng/ml of -0.05 and lower 95% CI at most -0.08 and upper at most -0.02.

Table 8.2: Serum periostin levels at time points during study

Asthma

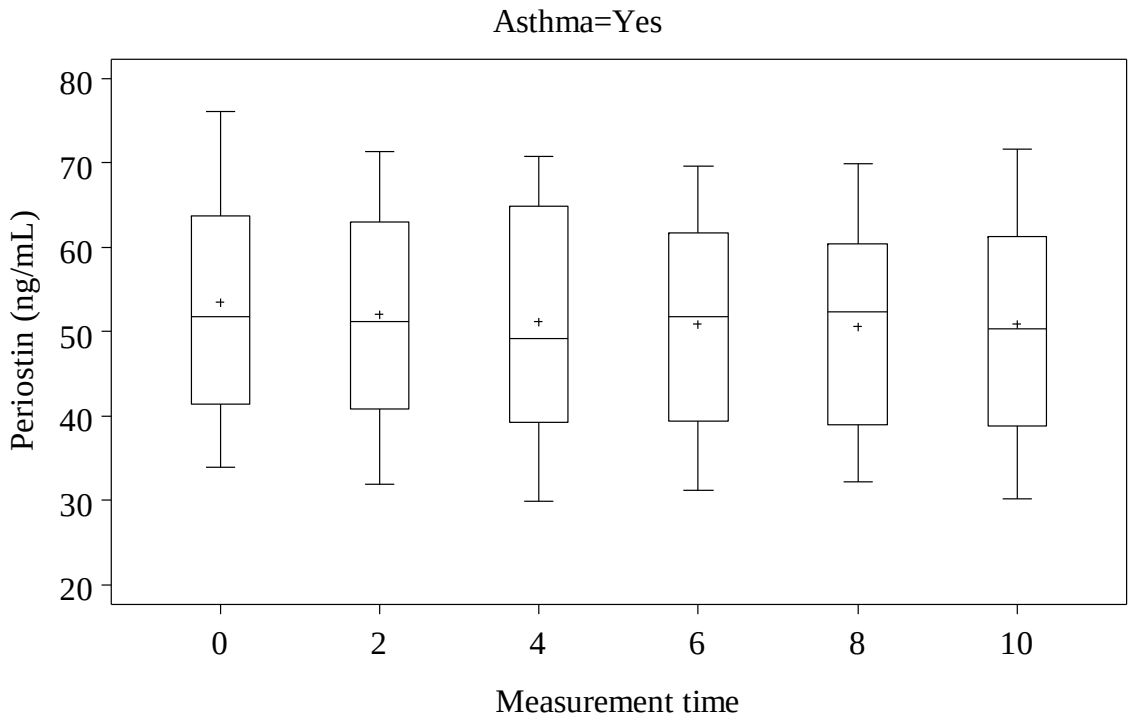
TIME POINTS	PERIOSTIN				
	Mean (SD)	Median (IQR)	Min to Max	Difference from baseline log periostin	Overall P value <0.001
0 (0800)	53.5 (13.6)	51.7 (41.5 to 63.7)	33.9 to 76.2		
2 (1000)	52.1 (12.5)	51.3 (40.8 to 63.0)	32 to 71.4	-0.02 (-0.05 to 0.003)	0.08
4 (1200)	51.3 (13.7)	49.3 (39.3 to 64.8)	29.9 to 70.8	-0.05 (-0.07 to -0.02)	<0.001
6 (1400)	51.0 (12.7)	51.7 (39.4 to 61.7)	31.2 to 69.6	-0.05 (-0.07 to -0.02)	<0.001
8 (1600)	50.6 (12.4)	52.3 (38.9 to 60.4)	32.3 to 70.0	-0.05 (-0.08 to -0.03)	<0.001
10 (1800)	50.9 (13.4)	50.3 (38.9 to 61.2)	30.3 to 71.6	-0.05 (-0.08 to -0.03)	<0.001

Table 8.2: Serum periostin levels at time points in study (continued)

Non-Asthma

TIME POINTS	PERIOSTIN				
	Mean (SD)	Median (IQR)	Min to Max	Difference from baseline log periostin	Overall P value <0.001
0 (0800)	50.5 (13.0)	49.4 (42.5 to 62.7)	28.6 to 70.5		
2 (1000)	50.3 (13.4)	48.6 (39.7 to 61.2)	28.7 to 72.7	-0.006 (-0.04 to 0.02)	0.71
4 (1200)	47.7 (12.2)	46.3 (39.5 to 59.7)	27.7 to 67.0	-0.06 (-0.09 to -0.03)	<0.001
6 (1400)	46.6 (11.3)	45.0 (39.8 to 53.8)	28.6 to 65.5	-0.08 (-0.11 to -0.05)	<0.001
8 (1600)	46.5 (11.9)	43.8 (38.6 to 55.9)	27.9 to 68.2	-0.08 (-0.11 to -0.05)	<0.001
10 (1800)	46.2 (11.5)	46.6 (37.7 to 55.0)	29.2 to 66.3	-0.08 (-0.11 to -0.06)	<0.001

Figure 8.2: Daytime Variation of Serum Periostin Levels in Asthma



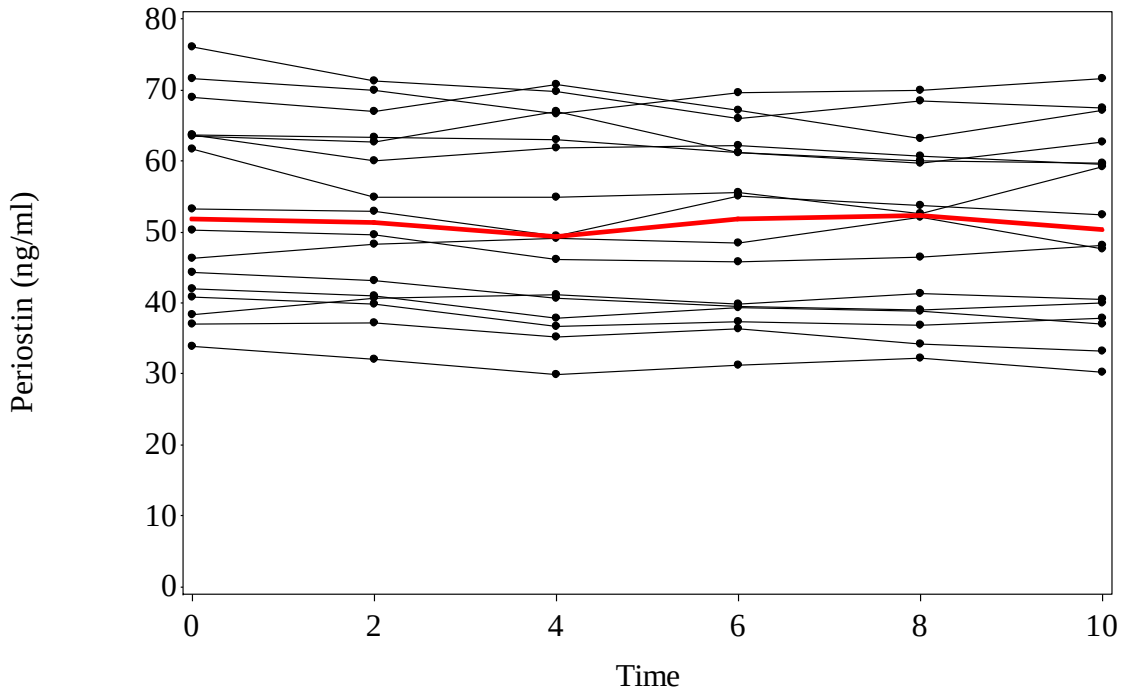
The box plots show the 25th, median and 75th percentiles as horizontal lines, the symbol is the mean, and the whiskers extend from the minimum to maximum values.

Table 8.3: Periostin Data in Participants with Asthma

Periostin (ng/ml)					
Baseline	**2 Hours**	**4 hours**	**6 Hours**	**8 Hours**	**10 Hours**
76.16	71.36	69.83	65.99	68.55	67.43
63.7	60.03	61.92	62.2	60.66	59.61
46.28	48.2	49.09	48.39	52.07	47.64
33.89	32	29.85	31.16	32.25	30.25
42.03	40.95	37.92	39.3	38.85	36.99
69.05	66.95	70.79	67.11	63.24	67.24
40.87	39.9	36.68	37.38	36.91	37.79
44.31	43.12	40.7	39.48	38.99	40.02
37.02	37.16	35.23	36.33	34.22	33.16
53.22	52.99	49.42	55.1	53.75	52.4
71.6	70	66.59	69.64	69.97	71.61
63.68	63.29	63.1	61.28	60.11	59.79
61.63	54.9	54.94	55.54	52.54	59.2
50.26	49.53	46.06	45.8	46.39	48.15
38.39	40.63	41.18	39.81	41.39	40.51
63.52	62.61	66.93	61.16	59.75	62.65

Participant highlighted would change classification from ‘high Periostin’ to ‘low Periostin’ based on the baseline and 10 hour periostin levels, utilizing the proposed periostin cut point of 50 ng/ml.

Figure 8.3: Individual joined line plots for Periostin in Asthma group



(— Median Periostin)

The FeNO decreased during the day from a peak mean of 26.1 ppb at 0800 hours to lowest level of 21.7 ppb at 1800 hours. There was strong evidence, overall P <0.001, that the means by time were different, shown in Table 8.4, Figure 8.4, Table 8.5, and Figure 8.5. Compared with baseline, the log FeNO was statistically significantly lower from the 6-hour (1400 hours) to the 10-hour (1800 hours) time points. The size of the difference from the 6-hour time point ranged from an estimate difference (95%CI) of -0.16 (-0.26 to -0.05) to -0.22 (-0.33 to -0.12).

Table 8.4: FeNO levels at time points during study

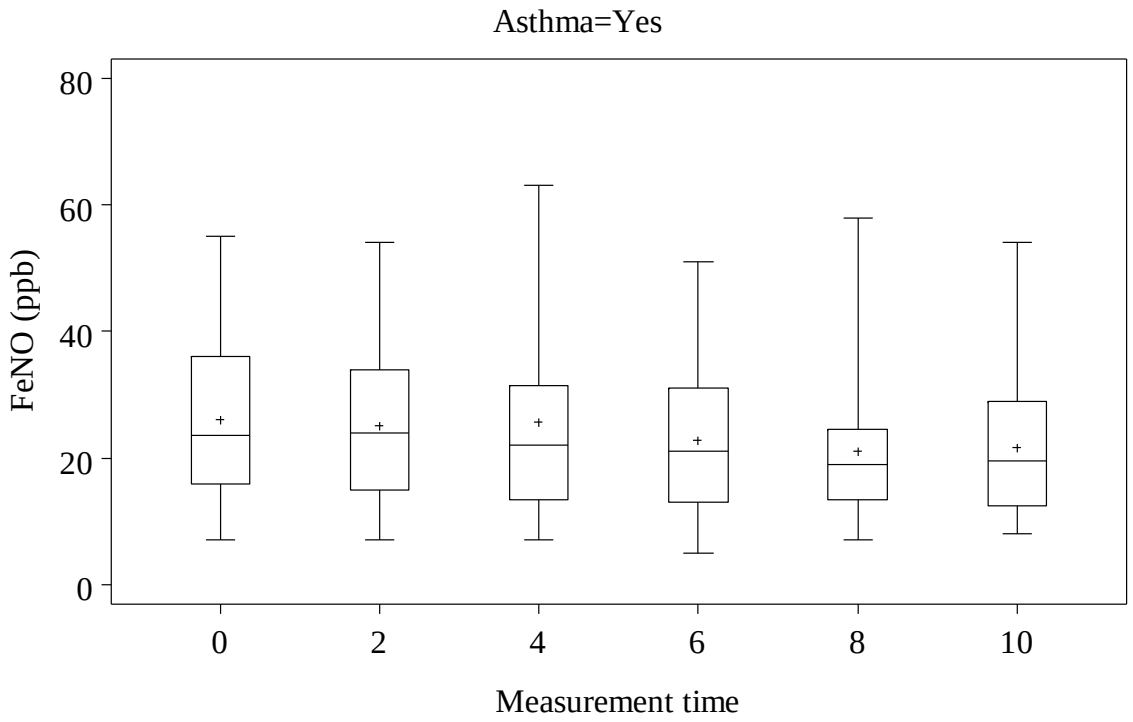
Asthma

TIME POINTS	FeNO				
	Mean (SD)	Median (IQR)	Min to Max	Difference from baseline log periostin	Overall P value <0.001
0 (0800)	26.1 (13.3)	23.5 (16 to 36)	7.0 to 55.0	-	-
2 (1000)	25.1 (13.3)	24.0 (15 to 34)	7.0 to 54.0	-0.04 (-0.15 to 0.07)	0.44
4 (1200)	25.8 (16.7)	22.0 (13.5 to 31.5)	7.0 to 63.0	-0.05 (-0.16 to 0.05)	0.32
6 (1400)	22.8 (12.4)	21.0 (13 to 31)	5.0 to 51.0	-0.16 (-0.26 to -0.05)	0.005
8 (1600)	21.3 (12.9)	19.0 (13.5 to 24.5)	7.0 to 58.0	-0.22 (-0.33 to -0.12)	<0.001
10 (1800)	21.7 (12.1)	19.5 (12.5 to 29)	8.0 to 54.0	-0.18 (-0.29 to -0.07)	0.001

Non-Asthma

TIME POINTS	FeNO				
	Mean (SD)	Median (IQR)	Min to Max	Difference from baseline log periostin	Overall P value <0.004
0 (0800)	22.3 (14.1)	19.5 (15.5 to 24.5)	6.0 to 68.0	-	-
2 (1000)	23.6 (14.9)	19.5 (16.5 to 27.5)	6.0 to 73.0	0.06 (-0.05 to 0.18)	0.30
4 (1200)	21.3 (13.9)	17.0 (13.5 to 27.0)	7.0 to 66.0	-0.05 (-0.17 to 0.06)	0.38
6 (1400)	22.8 (18.6)	16.0 (15.0 to 26.0)	7.0 to 88.0	-0.02 (-0.14 to 0.09)	0.72
8 (1600)	20.9 (15.4)	17.0 (13.0 to 23.5)	6.0 to 72.0	-0.10 (-0.22 to 0.01)	0.09
10 (1800)	18.5 (11.2)	16.0 (13.0 to 21.5)	6.0 to 56.0	-0.17 (-0.28 to -0.05)	0.005

Figure 8.4: Daytime Variation of FeNO in Asthma



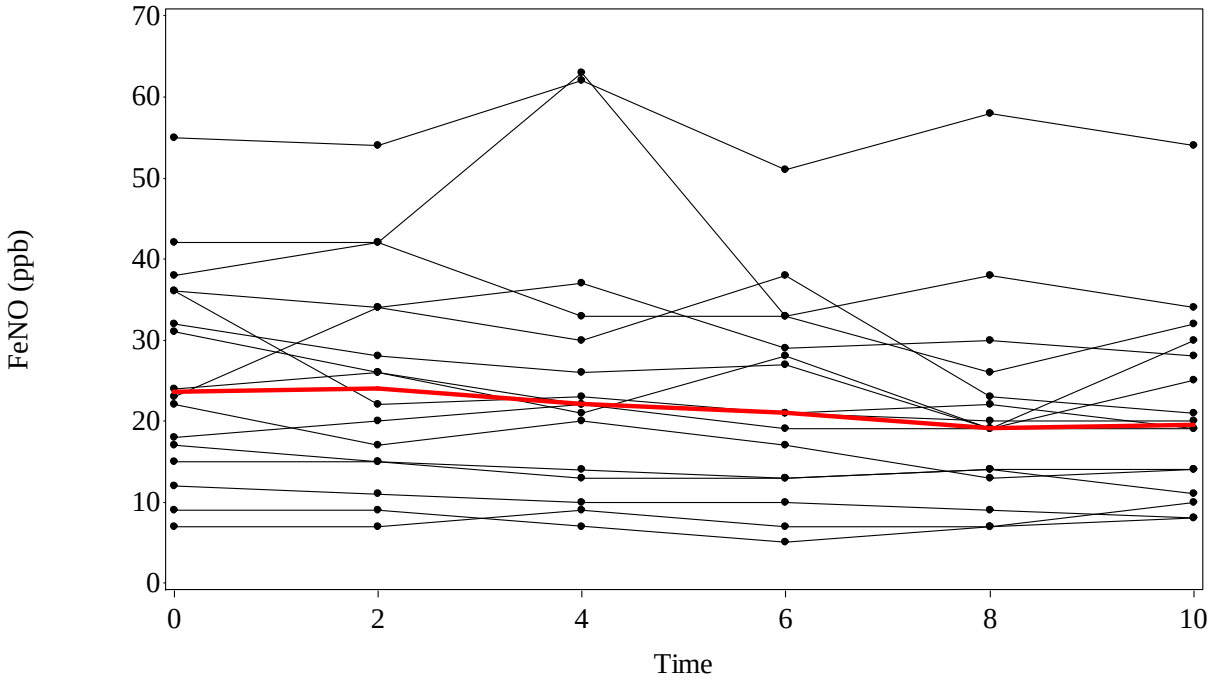
The box plots show the 25th, median and 75th percentiles as horizontal lines, the symbol is the mean, and the whiskers extend from the minimum to maximum values.

Table 8.5: FeNO Data in Participants with Asthma

FeNO (ppb)					
Baseline	**2 Hours**	**4 hours**	**6 Hours**	**8 Hours**	**10 Hours**
55	54	62	51	58	54
7	7	9	7	7	8
36	22	23	21	20	20
31	26	21	28	19	30
18	20	22	19	19	19
15	15	14	13	14	11
36	34	37	29	30	28
32	28	26	27	19	25
42	42	33	33	38	34
9	9	7	5	7	10
38	42	63	33	26	32
22	17	20	17	13	14
17	15	13	13	14	14
12	11	10	10	9	8
23	34	30	38	23	21
24	26	22	21	22	19

Participants highlighted would change classification from ‘high FeNO’ to ‘low FeNO’ based on the baseline and 10 hour FeNO levels, utilizing the proposed FeNO cut point of 19.5ppb.

Figure 8.5 Individual joined line plots for FeNO in Asthma Group



(— Mean FeNO)

There was some evidence, P=0.015, that the mean blood eosinophil count differed by time during the 10-hour period of the study (Table 8.6) but none of the individual comparisons with baseline were statistically significant.

Table 8.6: Blood eosinophil (x 10^9/L) levels at time points during study

Asthma

TIME POINTS	BLOOD EOSINOPHIL				
	Mean (SD)	Median (IQR)	Min to Max	Difference (95% CI) from baseline	Overall P value =0.015
0 (0800)	0.31 (0.20)	0.25 (0.20 to 0.35)	0 to 0.7	-	-
2 (1000)	0.31 (0.20)	0.20 (0.20 to 0.40)	0.10 to 0.7	0.006 (-0.03 to 0.04)	0.71
4 (1200)	0.29 (0.20)	0.20 (0.15 to 0.40)	0.10 to 0.7	-0.02 (-0.05 to 0.01)	0.26
6 (1400)	0.29 (0.21)	0.2 (0.1 to 0.4)	0.1 to 0.8	-0.02 (-0.05 to 0.01)	0.26
8 (1600)	0.31 (0.19)	0.25 (0.20 to 0.40)	0.1 to 0.7	0 (-0.03 to 0.03)	0.99
10 (1800) N=15	0.34 (0.21)	0.30 (0.20 to 0.40)	0.1 to 0.8	0.02 (-0.01 to 0.06)	0.20

Non-Asthma

TIME POINTS	BLOOD EOSINOPHIL				
	Mean (SD)	Median (IQR)	Min to Max	Difference (95% CI) from baseline	Overall P value <0.001
0 (0800)	0.19 (0.11)	0.20 (0.10 to 0.25)	0.10 to 0.50	-	-
2 (1000)	0.16 (0.10)	0.10 (0.10 to 0.20)	0.20 to 0.50	-0.04 (-0.07 to -0.01)	0.023
4 (1200) N=15	0.13 (0.10)	0.10 (0.10 to 0.20)	0 to 0.4	-0.06 (-0.09 to -0.02)	0.001
6 (1400)	0.14 (0.10)	0.10 (0.10 to 0.20)	0.0 to 0.3	-0.06 (-0.09 to -0.02)	<0.001
8 (1600)	0.15 (0.08)	0.10 (0.10 to 0.20)	0.10 to 0.30	-0.04 (-0.08 to -0.01)	0.008
10 (1800) N=15	0.17 (0.09)	0.10 (0.10 to 0.20)	0.10 to 0.40	-0.03 (-0.06 to 0.01)	0.13

FEV_1 % predicted progressively decreased during the day from 90.5% at 0800 hours to 85.8% at 1800 hours. No participant received SABA for relief during the 10-hour period of the study

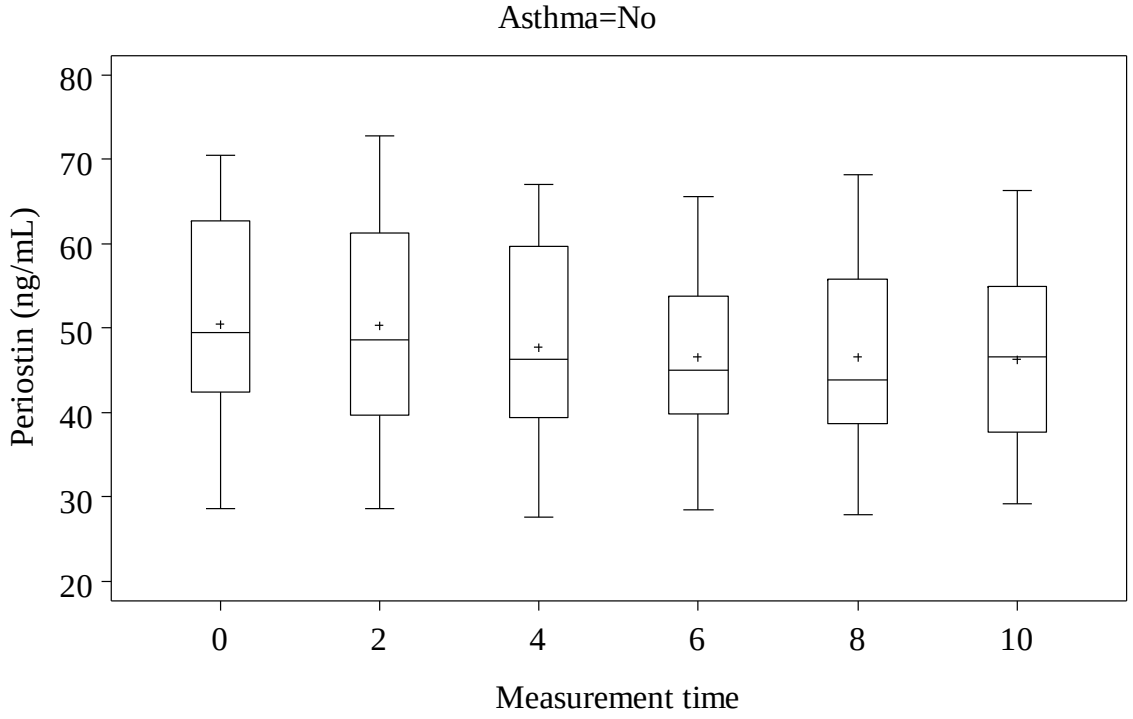
In a post hoc analysis of the asthma group, there was a change in classification from 'high periostin' to 'low periostin', based on the 0800 and 1800 hour periostin levels, utilizing the proposed cut point of 50 ng/ml in 1/16 participants (50.3 ng/ml and 48.2 ng/ml at 0800 and 1800 hours, respectively) (Table 8.3). There was no change in classification from 'low periostin' to 'high periostin', using the same criteria.

There was a change in classification from 'high FeNO' to 'low FeNO', based on the 0800 and 1800 hour FeNO levels, utilizing the proposed cut point of 19.5 ppb in 2/16 (Table 8.5). There was no change in classification from 'low FeNO' to 'high FeNO' using the same criteria.

Day-time changes in Non-Asthma

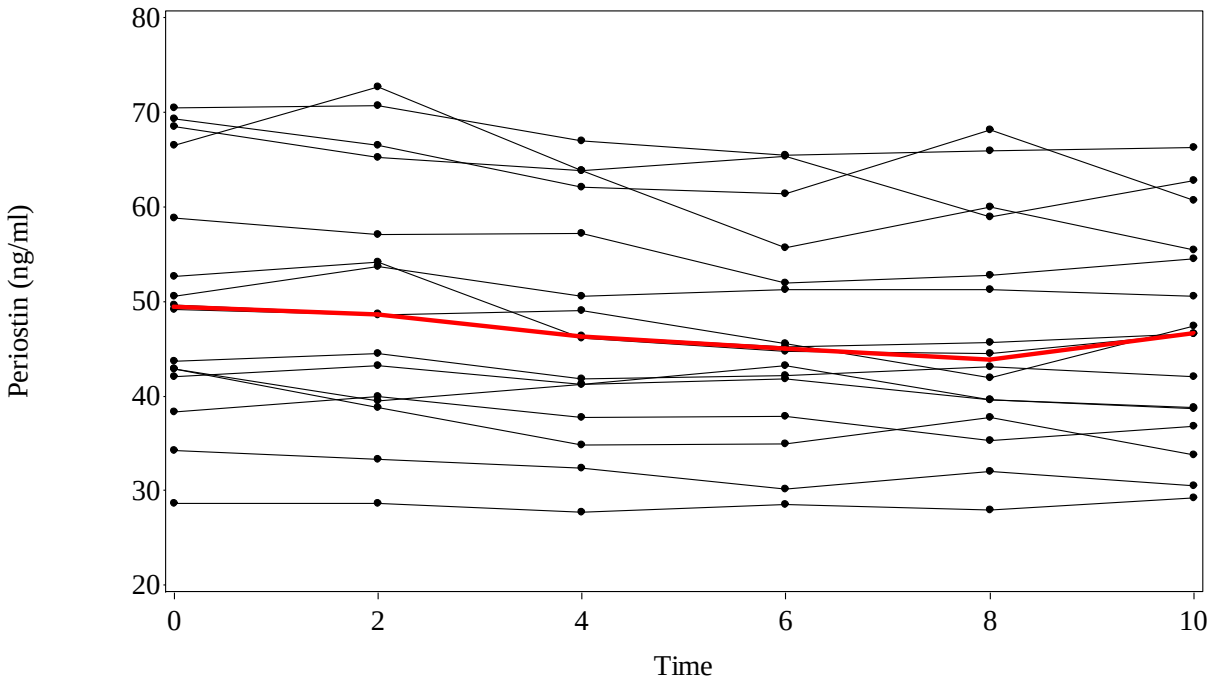
Serum periostin level progressively decreased during the day from a mean of 50.5 ng/ml at 0800 hours to 46.2 ng/ml at 1800 hours. There was strong evidence, overall $P < 0.001$, that the means by time were different, shown in Table 8.2, Figures 8.6 and 8.7 and Table 8.7. The magnitude of the difference compared with baseline was, as for the asthma group, stable from the 4-hour (1200 hours) to the 10-hour (1800 hours) time points. The size of the estimated difference (95% CI) in log periostin ng/ml from the 4-hour time point ranged from -0.06 (-0.09 to -0.03) to -0.08 (-0.11 to -0.06).

Figure 8.6: Daytime Variation of Periostin in Non-Asthma



The box plots show the 25th, median and 75th percentiles as horizontal lines, the symbol is the mean, and the whiskers extend from the minimum to maximum values.

Figure 8.7: Individual joined line plots for Periostin in Non-Asthma group



(— Mean Periostin)

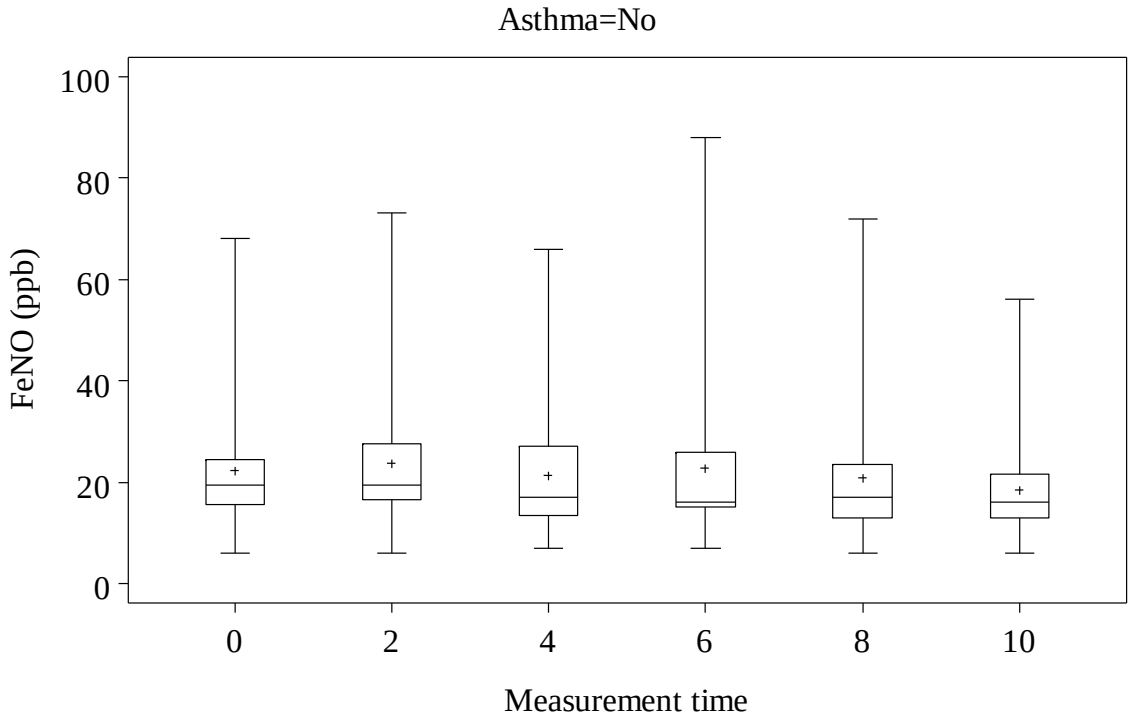
Table 8.7: Periostin Data in Participants without Asthma

Periostin (ng/ml)					
Baseline	**2 Hours**	**4 hours**	**6 Hours**	**8 Hours**	**10 Hours**
58.84	57.14	57.23	51.99	52.79	54.58
42.09	43.22	41.25	43.27	39.56	38.62
50.55	53.67	50.51	51.3	51.31	50.57
49.22	48.53	49.09	45.59	41.96	47.36
66.51	72.73	63.88	55.66	60.01	55.41
70.46	70.76	66.98	65.54	65.93	66.25
49.64	48.71	46.34	45.21	45.71	46.63
38.3	39.91	37.69	37.87	35.32	36.77
68.46	65.27	63.84	65.35	58.96	62.75
43.68	44.5	41.83	42.21	43.13	42.07
42.81	38.78	34.77	34.92	37.71	33.83
28.61	28.67	27.68	28.55	27.89	29.22
69.35	66.54	62.13	61.35	68.16	60.75
42.88	39.44	41.23	41.82	39.57	38.75
34.21	33.36	32.39	30.14	32.02	30.48
52.62	54.13	46.16	44.79	44.54	46.59

Participant highlighted would change classification from 'high Periostin' to 'low Periostin' based on the baseline and 10 hour periostin levels, utilizing the proposed periostin cut point of 50 ng/ml.

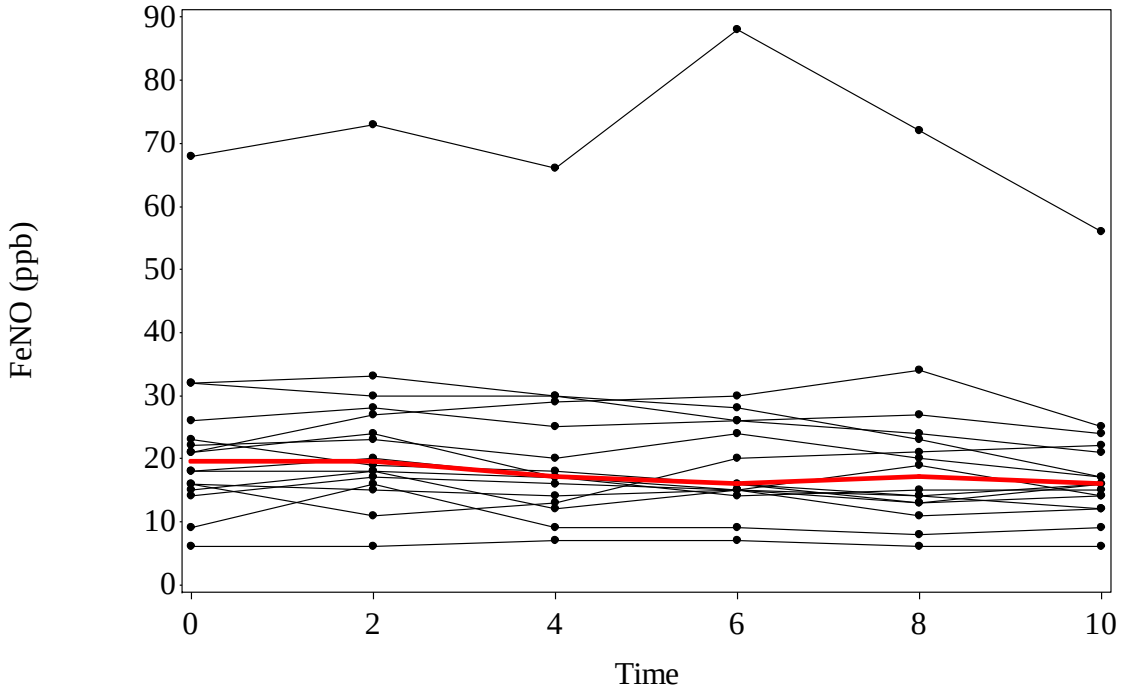
FeNO progressively decreased during the day from 22.3 ppb at 0800 hours to 18.5 ppb at 1800 hours. Again there was strong evidence, overall P=0.004, that the means by time were different, shown in Table 8.4, Figures 8.8, 8.9 and Table 8.8. Compared with baseline, the log FeNO was statistically significantly lower from the 10-hour (1800 hours) time point with an estimated difference (95%CI) of 0.17 (-0.28 to -0.05; P=0.005).

Figure 8.8: Daytime Variation of FeNO in Non-Asthma



The box plots show the 25th, median and 75th percentiles as horizontal lines, the symbol is the mean, and the whiskers extend from the minimum to maximum values.

Figure 8.9: Individual joined line plots for FeNO in Non-Asthma Group



(— Median FeNO)

Table 8.8: FeNO Data in Participants without Asthma

FeNO (ppb)					
Baseline	**2 Hours**	**4 hours**	**6 Hours**	**8 Hours**	**10 Hours**
68	73	66	88	72	56
16	11	13	20	21	22
22	23	20	24	20	17
16	15	14	15	19	14
18	18	17	16	14	16
32	30	30	28	23	17
21	27	29	30	34	25
18	20	17	14	15	15
21	24	17	15	11	12
26	28	25	26	24	21
14	17	16	15	13	14
15	18	12	15	14	12
23	19	18	16	13	16
9	16	9	9	8	9
6	6	7	7	6	6
32	33	30	26	27	24

Participants highlighted would change classification from 'high FeNO' to 'low FeNO' based on the baseline and 10 hour FeNO levels, utilizing the proposed FeNO cut point of 19.5ppb.

Blood eosinophil count progressively decreased during the day from a mean of 0.19 x 10^9/L at 0800 hours to a nadir of 0.13 x 10^9/L. There was strong evidence that the means were different by time, overall P<0.001, as shown in Table 8.6. Compared with baseline, the blood eosinophil count was statistically significantly lower at the 2, 4-, 6- and 8-hour time points with estimated differences (95%CI) ranging from -0.06 (-0.09 to -0.02) to -0.04 (-0.08 to -0.01).

9

DISCUSSION

This study shows that there is detectable variation in serum periostin levels through the day in adults with and without asthma. In both groups the serum periostin level was higher in the morning and lower in the afternoon. However, the magnitude of the variation was small and the proportion of asthma patients that changed classification from 'high periostin' to 'low periostin' during the day was 1 in 16, suggesting that the time of day at which serum periostin levels are measured is unlikely to influence treatment decisions if serum periostin levels are used to predict responsiveness to or eligibility for monoclonal antibody therapy directed against IL-13, IgE or possibly other components of Type-2 inflammation in asthma.

The findings are consistent with the distribution of serum periostin in health and respiratory disease in which the same periostin test was used. The median baseline values of 51.7 ng/ml and 49.4 ng/ml in the asthma and non-asthma groups respectively are similar to the median levels of 50.1 ng/ml in a population without asthma or COPD, as reported in Study A of this thesis, 53.7 ng/ml in a random adult population with a diagnosis of asthma (Fingleton et al. submitted), and 50.2 ng/ml in adults with moderate to severe asthma inadequately controlled despite ICS therapy (Corren et al. 2011). While recognizing that serum periostin is reduced with ICS therapy (Fingleton et al. submitted), these findings suggest that serum periostin is not a measure which can differentiate patients with asthma across a range of severity from a population without asthma.

Secondly, there was a wide range in serum periostin levels observed in both the asthma and non-asthma groups (34 to 76 ng/ml and 29 to 70 ng/ml respectively). This compares with a range of 15 to 165 ng/ml in an adult population with obstructive airways disease (Fingleton et al. submitted) and 28 to 136 ng/ml in an adult population without asthma or COPD (described in the first study in this thesis). The greater range in the previous studies is likely to be attributed at least in part to the larger numbers studied (N=285 and N=480, respectively).

Thirdly, the daytime variation with serum periostin levels was similar to that reported previously for the Type2-related biomarkers of sputum and blood eosinophils (Kelly et al. 2004; Panzer et al. 2003; Uhrbrand 1958; Wempe et al. 1992; Winkel et al. 1981) and FeNO (Pijnenburg et al. 2006; Saito et al. 2014; ten Hacken et al. 1998). In allergic subjects with mild asthma, sputum eosinophils are about two-fold higher at 0700 hours than at 1600 hours (Panzer et al. 2003). Additionally, the early morning increase in sputum eosinophils correlated with enhanced airway obstruction and reversibility, suggesting that airway recruitment of eosinophils might contribute to circadian variations in lung function in patients with asthma.

In adults with mild asthma, blood eosinophil counts were about 25% higher at 0400 hours than at 1600 hours (Kelly et al. 2004). It has been observed that the circadian change in blood eosinophils and lung function appear to fall into a continuous range, suggesting that day/night variations in airways inflammation and lung function occur as a continuum, rather than as an all or nothing phenomenon Kelly et al. 2004). In allergic subjects with moderately severe asthma, a circadian variation in blood eosinophil counts was also observed with peak values overnight (Wempe et al. 1992). In healthy subjects, blood eosinophil counts may also vary diurnally, being lowest in the morning and highest at night, correlating inversely to blood cortisol levels (Winkel et al. 1981). The demonstration of daytime variation in the non-asthma, but not the asthma group in our study is likely to reflect the lack of power, low sensitivity of the automated measurement of blood eosinophil levels to increments of 0.1 per 10^9/L, and possibly the effect of the maintenance treatment with ICS in all asthma participants.

In asthma, there is a variable degree of diurnal variation in FeNO, which is greatest in uncontrolled disease and serves as a predictor of risk of future exacerbations (Pijnenburg et al. 2006; Saito et al. 2014; ten Hacken et al. 1998). The mean diurnal FeNO variation, measured as the difference in morning (0700 hours to 1000 hours) from evening (1800 hours to 2100 hours) levels over a two-week period, was 15.6 ppb in uncontrolled asthma subjects compared with 8.2 ppb in stable controlled asthma, and 6.1 ppb in healthy subjects (Saito et al. 2014). In another study of adults with asthma, morning FeNO levels were reported to be 14% higher than evening levels (Pijnenburg el al. 2006). In this study it was observed a mean difference in FeNO between 0800 and 1800 hours on a single day of 4.4 ppb in controlled asthma and 3.8 ppb in non-asthma.

Finally, the lesser variability of serum periostin levels compared with FeNO levels in this study is consistent with previous observations that there is a lesser intra-patient variability in periostin levels compared with FeNO (Corren et al. 2011).

The clinical relevance of the findings in this study – in terms of no clinically important daytime variation in serum periostin – is suggested by the post hoc analysis in which we determined the proportion of patients that would have changed their classification of 'high' and 'low' periostin groups based on the 0800 and 1800 hour periostin levels, utilizing the proposed periostin cut point of 50 ng/ml, used to determine responsiveness to monoclonal antibody therapy directed against IL-13 (Corren et al 2011), and IgE (Hanania et al. 2013). There was only one of 16 asthma participants who changed their periostin classification, indicating that timing of periostin measurement during the day is unlikely to be an important consideration if the validity of this cut point as a predictor of responsiveness is confirmed in future studies. There were two of 16 asthma participants who changed classification based on the FeNO cut point of 19.5 ppb, proposed as a predictor of responsiveness to anti-IgE monoclonal antibody therapy (Hanania et al. 2013).

There are a number of methodological issues relevant to the interpretation of the study findings. Firstly, no adjustment has been made for multiple statistical testing, and as a result the findings should be considered illustrative. The findings are generalizable to adults with asthma on regular ICS and LABA treatment, representing Step 3 and 4 therapy) (Global Initiative for Asthma 2015). It is known that ICS reduce serum periostin levels (Fingelton et al. submitted), FeNO ((Pijnenburg et al. 2006; Saito et al. 2014; ten Hacken et al. 1998) and blood eosinophil counts (Wempe et al. 1992), however the effect of ICS on the circadian rhythm of periostin is not known. It is possible that serum periostin levels may show a greater magnitude of circadian variability in a population in which there is a greater proportion with uncontrolled disease, similar to an effect shown with FeNO (Saito et al. 2014). The use of LABA therapy prior to the baseline morning measurements and no subsequent medication use during the period of the study, is unlikely to have influenced the periostin or FeNO levels, but may have resulted in the gradual reduction in FEV_1 during the study period as the bronchodilator effect of the LABA wore off.

This study was limited to periostin levels across a single 10-hour period rather than a full 24-hour period which incorporated overnight measurements. However, in a clinical inpatient or outpatient situation it is likely to expect blood samples to be drawn for measurement of periostin between 0700 and 1800, the period included in this study. It would be useful to study the day to day variability in periostin levels to

further guide its use in clinical practice. The participants studied were primarily New Zealand European and thus the results are likely to be generalizable to Caucasian populations.

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CONCLUSION

It can be concluded from this study that there is day-time variation of serum periostin in adults with asthma receiving maintenance ICS and LABA therapy, with higher levels in the morning, as well as a day-time variation in adults without asthma or other respiratory disease. However, the magnitude of the variation in serum periostin levels is considered to not be clinically relevant, such that the time of day at which the blood sample is drawn is unlikely to influence treatment decisions if a specific serum periostin level is used to predict treatment responsiveness.

Part IV

SUMMARY OF FINDINGS AND POTENTIAL FUTURE RESEARCH

11

SUMMARY OF FINDINGS AND POTENTIAL FUTURE RESEARCH

Summary of Findings

- The 90% confidence interval for serum periostin levels in adults without asthma or COPD is 35.0 to 71.1 ng/ml.
- Serum Periostin values need not be adjusted for age, sex or common comorbidities in the adult population.
- Current smoking status is associated with lower serum periostin levels.
- There was a day-time variation in serum periostin levels in adults with asthma treated with ICS and LABA therapy, with higher values in the morning than in the afternoon.
- The time of day at which serum periostin levels are measured is unlikely to influence treatment decisions if a specific periostin level is used to predict responsiveness to, or eligibility for monoclonal antibody therapy directed against IL-13, IgE or other components of Type-2 inflammation in asthma.
- Similar small variations in serum periostin levels were observed in adults with and without asthma.

Potential Future Research

It is evident from the literature review conducted for this thesis that much more is known about the mechanisms for the regulation, induction and secretion of periostin at a cellular pathway level than the factors regulating periostin at a serum level. As a result there are a number of areas that would benefit from further research to fully evaluate serum periostin as a potential biomarker.
The analysis of ethnicity and serum periostin during the determination of serum reference values identified that those of Asian descent have a higher level of periostin; it would be advantageous to determine the reference range for serum periostin in an Asian population to quantify this difference.
The relationship between serum periostin and creatinine, acute and chronic renal failure would also be an essential area to further explore. The serum response to pathologic processes where periostin is

upregulated at a cellular level is another area to explore to fully assess periostin as a biomarker. Serum periostin levels in both the acute phase and throughout healing in response to bone fractures, joint replacements (where a baseline pre-injury serum level could be measured), dental extraction and myocardial infarction would be a valuable addition to the understanding of serum periostin.
As asthma is an increasing and prominent disease worldwide, evaluating serum periostin as a potential biomarker to direct therapy and monitor treatment would be extremely valuable. Further research to determine the effect of asthma on serum periostin would need to include; evaluating the stability of periostin in stable asthmatics over a period of time (daily and weekly serial measurements), as well as determining the effects of an exacerbation of asthma requiring oral steroid treatment on serum periostin acutely and during recovery.

Part V

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Part VI

APPENDIX

A

GENERAL HEALTH QUESTIONNAIRE

Periostin Reference Range General Health Questionnaire

Participant ID: | | | | | | | | |

Name:

Date: Day | Month | Year

A. General Questions

1. Date of Birth — Day | Month | Year

   Age

2. Which ethnic group do you belong to? Mark the space or spaces which apply to you.

   - Maori
   - New Zealand European
   - Samoan
   - Cook Island Maori
   - Tongan
   - Niuean
   - Chinese
   - Indian
   - Other (such as *DUTCH, JAPANESE, TOKELAUAN*). Please state:

3. Are you male or female? M | F

B. Smoking History

4a. Have you ever smoked cigarettes? Yes | No

4b. Do you currently smoke cigarettes? Yes | No

4c. If NO, date of last cigarette: D D | M M | Y Y Y Y

4d. Number of years as smoker — Yrs ☐ N/A ☐

4e. Number of cigarettes smoked per day on average: — Cigarettes ☐ N/A ☐

Investigator calculate pack years — N/A ☐

C. Exercise

5. In the last 7 days have you exercised for >30minutes at a time and had to breathe hard. — Yes ☐ No ☐

If NO, please go to question 6, if YES, proceed with this section:

5a. Date of last exercise ☐☐ ☐☐ ☐☐☐☐

5b. Time of last exercise ☐☐☐☐

5c. Exercise Activity — Activity/Activities ☐

D. Respiratory

6a. Have you ever been diagnosed with asthma, chronic bronchitis, COPD, emphysema or bronchiectasis? — Yes ☐ No ☐

6b. Have you experienced any wheezing or whistling in the chest in the past 12 months? — Yes ☐ No ☐

6c. Have you ever been admitted to hospital or required surgery for a condition relating to your lungs? — Yes ☐ No ☐

Please Specify: ☐

E. Eyes

7a. Do you ever experience itchy or watery eyes? — Yes ☐ No ☐

Please Specify:

7b. Have you been diagnosed with an eye condition? Yes ☐ No ☐

Please Specify:

F. Ear, Nose and Throat

8a. Do you experience symptoms of Rhinitis (runny/stuffy, itchy nose, sneezing) not associated with a cold/flu? Please circle

Runny Nose | Stuffy Nose | Itchy nose | Sneezing

8b. Have you been diagnosed with nasal polyps? Yes ☐ No ☐

8c. Do you have a known tooth cavity? Yes ☐ No ☐

8d. Have you had a dental procedure in the last 3 months? Yes ☐ No ☐

8e. Have you been diagnosed with an ear, nose or throat condition? Yes ☐ No ☐

Please Specify:

G. Gastrointestinal

9a. Do you have Gastroesophageal Reflux Disease (GORD) or reflux? Yes ☐ No ☐

9b. Do you have eosinophilic oesophagitis? Yes ☐ No ☐

9c. Do you have inflammatory bowel disease (crohns disease, ulcerative colitis)? Yes ☐ No ☐

Please Specify:

9d. Have you been diagnosed with a condition relating to your digestive tract (oesophagus, stomach or bowel)? Yes ☐ No ☐

Please Specify:

H. Cardiovascular

		Yes	No
10a.	Have you been diagnosed with high blood pressure?		
10b.	Have you ever been diagnosed with high cholesterol?		
10c.	Do you experience chest pain/angina?		
10d.	Have you ever had a heart attack (myocardial infarction)?		
10e.	Have you ever been diagnosed with heart failure?		
10f.	Have you ever been diagnosed with problem with your heart valve(s)?		
10g.	Have you ever been admitted to hospital for a heart condition or had cardiac surgery?		

Please Specify:

I. Genitourinary

		Yes	No
11a.	Have you ever been diagnosed with kidney disease?		

Please Specify:

		Yes	No
11b.	FEMALES: Are you using contraception?		

Please Specify:

		Yes	No
11c.	FEMALES: Are you post- menopausal?		

Date of last Period:

J. Musculoskeletal

12a. Have you ever been diagnosed with osteoarthritis? Yes ☐ No ☐

12b. Have you ever been diagnosed with inflammatory arthritis? (rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis) Yes ☐ No ☐

12c. Have you ever been diagnosed with osteoporosis? Yes ☐ No ☐

12d. Have you ever broken a bone? Yes ☐ No ☐

Date of most recent Fracture:

Site of most recent Fracture:

12e. Have you ever had a joint replacement? Yes ☐ No ☐

Date of Replacement:

Site of Replacement:

K. Skin

13a. Have you ever been diagnosed with eczema? Yes ☐ No ☐

13b. Have you ever been diagnosed with psoriasis? Yes ☐ No ☐

13c. Do you have a current open wound? Yes ☐ No ☐

Date of Injury:

Site of Wound:

L. Neurological

14. Have you ever been diagnosed with a neurological condition? Yes ☐ No ☐

Please Specify:

M. Psychiatric

15. Have you ever been diagnosed with a psychiatric or mental health condition? Yes ☐ No ☐

Please Specify:

N. Endocrine

16a. Have you ever been diagnosed with a Thyroid condition? Yes ☐ No ☐

Please Specify:

16b. Have you ever been diagnosed with diabetes? Yes ☐ No ☐

16c. Have you ever been diagnosed with impaired glucose tolerance or prediabetes? Yes ☐ No ☐

16d. Have you ever been diagnosed with any other endocrine condition? Yes ☐ No ☐

Please Specify:

O. Haematology

17a. Have you ever been diagnosed with a blood disorder? e.g. anaemia, haemochromatosis, leukaemia Yes ☐ No ☐

Please Specify:

P. Cancer

18a. Have you ever been diagnosed with cancer? Yes ☐ No ☐

Please Specify site:

Date:

Treatment:

Q. Allergies

19. Do you have any allergies? Yes ☐ No ☐

Please Specify:

R. Medications

Investigator: **Please document all medications in visit worksheet**

Medications reconciled to medical history? Yes 1 ☐ No 0 ☐

B

JUSTIFICATION OF GENERAL HEALTH QUESTIONNAIRE

Periostin Reference Range General Health Questionnaire Justification for Questions

A. General Questions

1. Date of birth:	Demographic Information
2. Ethnic Group:	Demographic Information
3. Sex:	Demographic information

B. Smoking History

4a-f. Smoking history	General Health information

C. Exercise

5a-c. Exercise	Periostin is found in tendon and bone injury and repair (Sidhu et al. 2010) We hypothesise that exercise will cause stress to bones and ligaments and may have an effect on periostin production and serum levels.

D. Respiratory

6a-c. Respiratory Conditions	Exclusion criteria for this study. Periostin is a marker of airway eosinophilia and inflammation (Sidhu et al. 2010; Jia et al. 2012; Ingram et al. 2012; Gordon et al. 2011) Periostin decreases the destensibility of airways and plays a significant role in airway fibrosis (Sidhu et al. 2010; Jia et al. 2012; Takayama et al. 2006)

E. Eyes

7a. Do you ever experience watery eyes?	A symptom of allergic rhinitis or hayfever. Periostin levels have been found to be increased in TH2 mediated inflammation including allergic rhinitis (Jia et al. 2012; Ingram et al. 2012) To assist in interpretation of IgE result
7b. Have you been diagnosed with an eye condition?	General Health information

F. Ear nose and Throat

8a. Do you experience symptoms of Rhinitis?	Periostin levels have been found to be increased in TH2 mediated inflammation including allergic rhinitis (Jia et al. 2012; Ingram et al. 2012)
8b. Have you been diagnosed with nasal polyps?	TH2 mediated inflammation with increased periostin expression (Jia et al. 2012; Ingram et al. 2012)
8c and 8d. Do you have a known tooth cavity?	Periostin is found in periodontal ligaments, bone and tooth development, injury and repair (Sidhu et al. 2010).
8e. Have you ever been diagnosed with an ear, nose or throat condition?	General Health information

G. Gastrointestinal

9a. Do you have Gastroesophageal disease (GORD) or reflux?	Periostin levels are elevated in TH2 mediated inflammation which includes eosinophilic oesophagitis very commonly misdiagnosed as gastroesophageal reflux disease (Jia et al. 2012; Blanchard et al. 2008)
9b. Do you have eosinophilic oesophagitis?	TH2 mediated inflammation with increased periostin expression (Jia et al. 2012; Blanchard et al. 2008).
9c. Do you have inflammatory bowel disease?	Periostin plays a role in the inflammatory response of epithelial cells including in epithelial injury as seen in inflammatory bowel disease (Ontsuka et al. 2012)
9d. Have you been diagnosed with a condition relating to your digestive tract?	General Health information

H. Cardiovascular

10a-e.General health information, cardiovascular risk factors and history of myocardial infarction and heart failure?	Periostin is required for embryonic myocardial development and repair of damage to myocardium death of heart muscle (myocardial infarction/heart attack) and heart failure (Sidhu et al. 2010; Blanchard et al. 2008)
10f. Have you ever been diagnosed with problem with your heart valve(s)?	Periostin is required for embryonic valve development and repair of damaged heart valves (Sidhu et al. 2010).
10g. Have you ever been admitted to hospital for heart condition or had cardiac surgery?	Assess severity

I. Genitourinary

11a. Have you ever been diagnosed with kidney disease?	Increased expression of periostin has been found in hypertensive nephropathy described as a critical marker of progression/reversal of this disease (Geurrot et al. 2012). To assist in interpretation of blood results taken during study.
11b-c. Contraception and menopause	General health information and related to hormone related bone density loss.

J. Musculoskeletal

12a-e. Musculoskeletal history	Periostin is required for bone development and in injury and repair (Sidhu et al. 2010; Nakazawa et al. 2004).

K. Skin

13a. Have you ever been diagnosed with eczema?	Atopic dermatitis (atopic eczema) involves TH2 mediated inflammation with increased periostin expression (Sidhu et al. 2010; Zhou et al. 2010; Hamilton 2008). To assist in interpretation of IgE result
13b. Have you ever been diagnosed with psoriasis?	Periostin expression has been found to be present in the lesions of patients with psoriasis (Kou et al. 2014; Arima et al 2015).
13c. Do you have an open wound?	Periostin is expressed in skin formation and repair and is found in higher levels within hypertrophic and keloid scars (Zhou et al. 2010).

L. Neurological

14. Have you ever been diagnosed with a neurological condition?	General health information

M. Psychiatric

15. Have you ever been diagnosed with a psychiatric or mental health condition?	General health information

N. Endocrine

16a. Have you ever been diagnosed with a thyroid condition?	General health information as common endocrine condition with cardiovascular effects in both hypo and hyper states.
16b. Have you ever been diagnosed with diabetes?	General health information and cardiovascular risk factor
16c. Have you ever been diagnosed with impaired glucose tolerance or prediabetes?	General health information and cardiovascular risk factor
16d. Have you ever been diagnosed with any other endocrine condition?	General health information

O. Haematology

17. Have you ever been diagnosed with a blood disorder?	General health information. To assist in interpreting blood results from study

P. Cancer

18. Have you ever been diagnosed with cancer?	Periostin is expressed by many tumours particularly those of epithelial origin. Periostin has also found to be expressed in higher levels in distant metastases (Sidhu et al. 2010; Kyutoku et al. 2012). Known active cancer would also be considered an exclusion.

Q. Allergies

19. Do you have any allergies?	General health information

R. Medications

20. Current prescribed medications and any others including over the counter medication taken in the 7 days prior to visit.	General health information and to be used to reconcile medical history.